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Online Catalogue of the Spiders (Arachnida: *Araneae*) of Pakistan

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Abstract: The present spiders (Arachnida: Araneae) of Pakistan's digital catalogue on the website will provide updated knowledge of the number of species, genera and families distributed in Pakistan. The current counts are 36 families, 119 genera and 165 species based on literature and intensive surveys since 2013. This website will ease the worldwide research for spider species' global biodiversity explorations and distributions. The Checklist of spiders (Arachnida: Araneae) of Pakistan is available at <http://www.spidersofpakistan.com/checklist.aspx>.

Keywords: Spiders, <http://www.spidersofpakistan.com/>, ICUN, Pakistan

1. INTRODUCTION

The spiders of Pakistan remain incompletely studied. Besides the diverse landscape and merge Oriental, Palearctic regions with highly diverse mountains closed unreached valleys of Himalaya, Hindukush and Karakoram. The frequent literature available in the last two decades describes species from British India preserved in UK museums. While faunistic exploration after partitions are currently preserved in European, Russian, Chinese and American Museums are well studied and their revisions provide comprehensive taxonomic evidence of modern taxonomy [1-32].

The lack of proper taxonomic work, taxa ranking, inventories, species validations, inappropriate tagging and insufficient preservation in proper natural history museums spiders (Arachnida: Araneae) of Pakistan is always argued and identified species created distribution and species zoogeographic extension hurdles [10-14, 33]. Dyal, 1935 has described more than 38 species on spiders of Lahore [32] but their holotypes and paratypes may almost be lost or neither record of preservation.

The recent work of the author with others coauthors has resulted in the present catalogue of a baseline study to understand spiders taxa in

hyperdiverse mountains of Himalaya, Hindukush and Karakoram of Pakistan [35-40]. The aims and objectives of the current catalogue are; 1) an online catalogue/checklist for the spiders' diversity of Pakistan, 2) the endemic spiders' species of Pakistan for ICUN and evolutionary studies, 3) a catalogue will ease the global spiders' inventories and National Species Specialist Group experts under the ICUN Rules (2012) for listing spiders species in Pakistan, and 4) catalogue will provide data for new to science species of spiders from Pakistan.

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Comparison of Host Expression Systems used for Efficient Recombinant Proteins Production

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Abstract: The marvels of DNA recombination technology have revolutionized the field of biotechnology. Several hormones, antibody subunits, vaccines, enzymes, and interferons are being produced at the industrial level, in suitable expression systems, under optimized conditions. For recombinant protein production, a range of expression systems are available such as bacteria, yeast, fungi, plant cells, insects and animal cells, etc. All recombinant proteins are naturally different from each other and various challenges are kept into consideration while choosing an expression system for their production. Every expression system has its advantages and limitations on the basis of which it can be considered or rejected for a particular protein production. Therefore, it is very significant to investigate the potential and limitations of several expression systems to choose the suitable one for particular protein production at an industrial scale. The optimization criteria of an expression system is evaluated on several factors such as productivity, efficiency, physiological characteristics, total cost, safety, convenience, and down-streaming conditions. *Escherichia coli* and *Saccharomyces cerevisiae* remained the organisms of choice to produce recombinant proteins for a long time, but now several other microorganisms are also being targeted to evaluate their efficiency toward recombinant protein production. Prokaryotic expression systems can be used to produce eukaryotic proteins as well however, the use of a eukaryotic expression system is preferable because it retains the structural, functional, and regulatory properties of therapeutic proteins. This review illustrates a brief view of a variety of expression systems, their efficiency, and limitations in recombinant protein production.

Keywords: DNA Recombinant Technology, Expression Systems, Cell Lines, Recombinant Proteins

1. INTRODUCTION

Proteins are the basic biological building blocks which play a key role in the metabolic machinery of all life forms. Some proteins perform structural roles while others act as biocatalysts i.e., enzymes which accelerate the metabolic rate. Proteins are the most vital biological molecules that play a key role in almost every function of the cell such as immune responses, cell adhesion, cell signaling, and cell cycle [1]. These biological molecules can now be produced at a commercial scale, all thanks to recombinant DNA technology. Recombinant DNA (rDNA) technology refers to the genetic manipulation in an organism's genetic material to induce desired characteristics in the organism or to produce desired products i.e., proteins. It was in 1973 when the first recombinant DNA molecule

was produced at the University of California San Francisco and Stanford University by the combined efforts of Paul Berg, Herbert Boyer, Annie Chang, and Stanley Cohen [2].

At first rDNA technology was suggested to foster agriculture and drug development, however, several unexpected difficulties hinder the achieving satisfactory results [2]. Till 1980 several products i.e., vaccines, hormones, and therapeutic proteins had been developed. The first ever developed therapeutic recombinant protein was insulin which was approved as safe in 1982 after which it became one of the global scale pharmaceutical products with a continuously increasing demand worldwide [2-4]. Besides the pharmaceutical sector, enzyme and agriculture industries also take benefit from recombinant DNA technology [4].

At present, several genetically engineered proteins are produced at an industrial scale. These proteins are used in different sectors i.e., enzyme industries, pharmaceutical industries, agriculture industries, etc. The products manufactured in these industries act as raw materials for many other fields such as diagnostics, medicine, nutrition, pharmacy, detergent, paper pulp, textiles, plastics, leather, and polymers [1, 5]. The development and innovation in recombinant DNA technology have evolved the strategy of treatment. Figure 1 demonstrates some key advantages of DNA technology in diagnostics, treatment, and improvement of the health quality of people. According to the Vantage Market Research report, the value of the global recombinant DNA technology market remained at 142.9 Billion USD and is expected to surpass 223 Billion USD by 2018 [6].

To fulfill the required need of production, various expression systems have been established [7]. Expression system refers to the host cell, providing the metabolic machinery for protein synthesis, and a transfected or modified DNA vector, responsible for providing the blueprints of the desired protein. The genetic code of vector DNA is transcribed into mRNA which interacts with host ribosomes and results in the translation of desired amino acid sequence. During the process of translation, transfer RNA and ribosomal RNA

also play their key roles of supplying amino acids and catalyzing the linkages between amino acids respectively.

Translation of protein is followed by the addition of posttranslational modifications (PTMs) in the molecules which are complex and different for prokaryotes and eukaryotes [8]. Prokaryotic expression systems can be used to produce eukaryotic proteins as well [9]; however, the use of a eukaryotic expression system is preferable because it retains the structural, functional, and regulatory properties of therapeutic proteins [10, 11].

The present review illustrates the recently available variety of expression systems for the production of beneficial recombinant proteins. Moreover, a comparison of the efficiency and benefits of these expression systems along with their potential to produce recombinant proteins at an industrial scale will also be discussed in this review.

2. CONSIDERATIONS TO CHOOSE AN EXPRESSION SYSTEM

Choosing the right expression system to express the protein of interest is an extremely vital approach as it affects the outcomes. There are several factors to be considered to choose an expression system: the

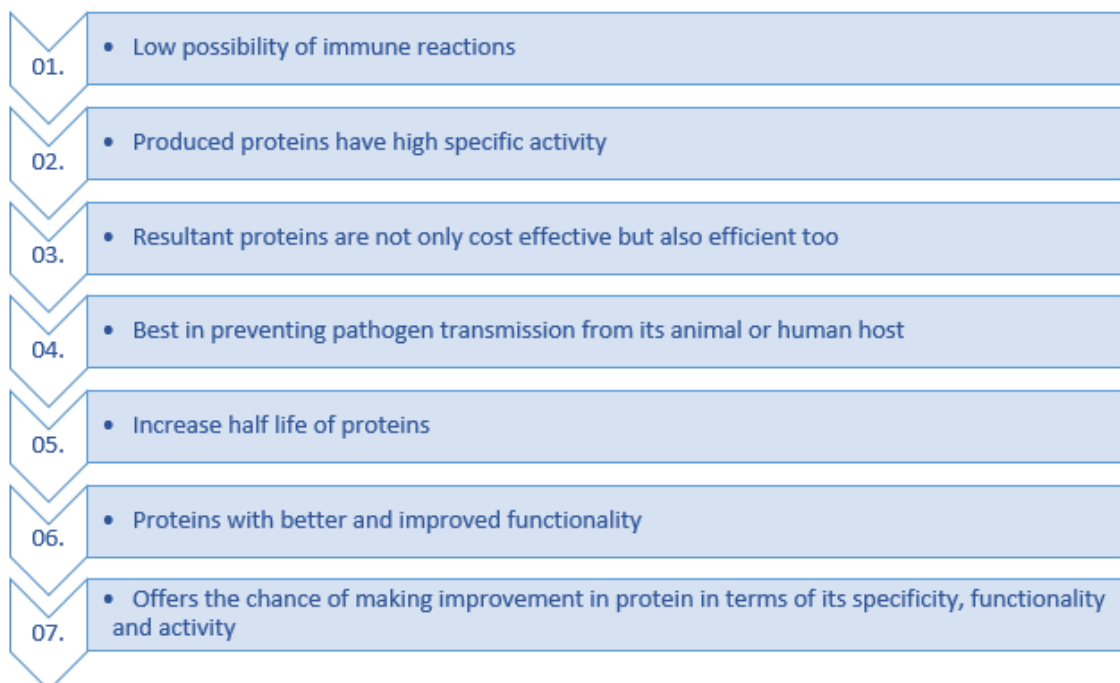


Fig. 1. Some advantages of using recombinant DNA technology in protein production

origin of the protein (prokaryotic or eukaryotic); solubility of the protein (insoluble proteins may form aggregates in the form of inclusion bodies or do not fold accurately); structural complexity, post-translational modifications, cellular localization, purification, yield and the total investment to produce the protein at commercial scale [12]. Every protein is different and requires different expression systems for proper recovery. Mostly, the recombinant proteins produced in an expression system behave foreign for it, hence the post-translational modifications could be different from the original product. Almost 5 % of the whole cell proteome consists of enzymes which perform several post-translational modifications which are different for genetically different types of cells [13]. It indicates that even if the host cell has a capability of a specific post-translation modification such as glycosylation, still there is a possibility of a difference in the pattern of glycosylation compared to the native protein. To retain the stability, immunogenicity, biological activity, and pharmacokinetic behavior of a therapeutic protein, its N-linked glycosylation pattern should be correct [14]. Some general considerations and requirements for the proficient production of recombinant protein are described in Figure 2.

2.1 Overview of Recombinant Proteins Production

Genetic manipulation involves the insertion of a specific DNA fragment, containing the gene of interest, in an appropriate vector. This process is facilitated by the enzymatic activity of two most important enzymes: endonucleases (for sequence-specific DNA cutting activity) and DNA ligases (to attach the gene of interest with the vector). The vector is further inserted into an expression system, grown to produce several copies of the gene of interest to produce desirable products [15] as demonstrated in Figure 3. There is a diversity of available expression system platforms for recombinant protein production. Different expression systems such as bacteria, yeast, mammalian cells, plants, and insects could be used. Among these expression systems, bacterial (*Escherichia coli*) and yeast expression systems (*Saccharomyces cerevisiae* and *Pichia pastoris*) are more common [16-17].

3. BACTERIA

Bacteria are simple unicellular prokaryotes which proves beneficial protein-producing cell factories. Bacteria are considered an easily available

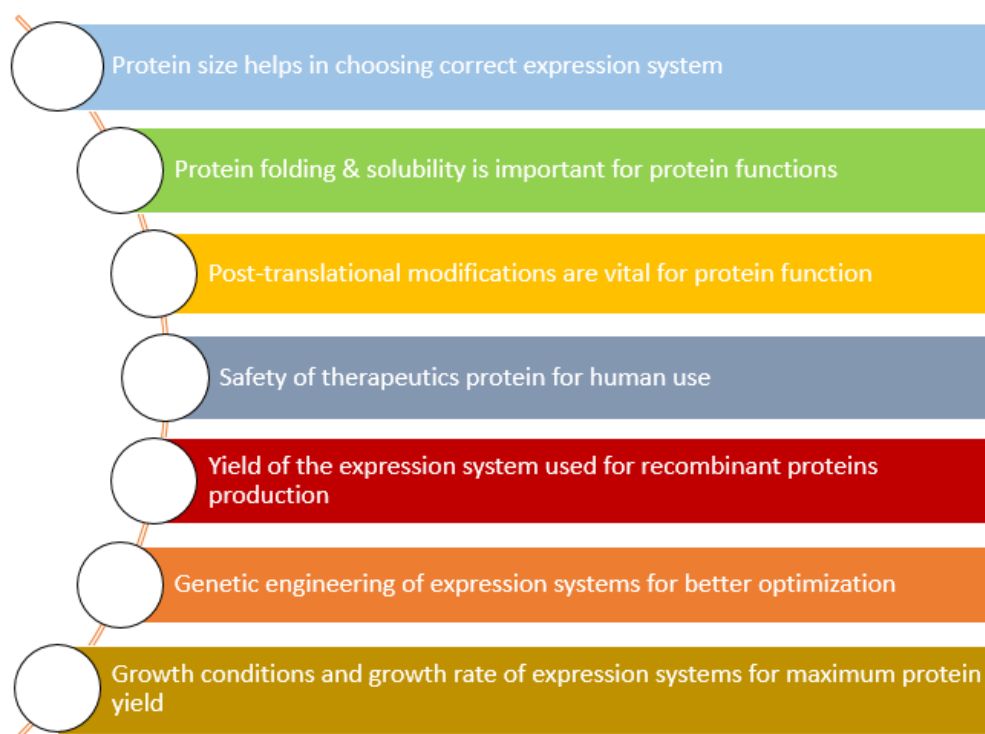


Fig. 2. General considerations for recombinant proteins production

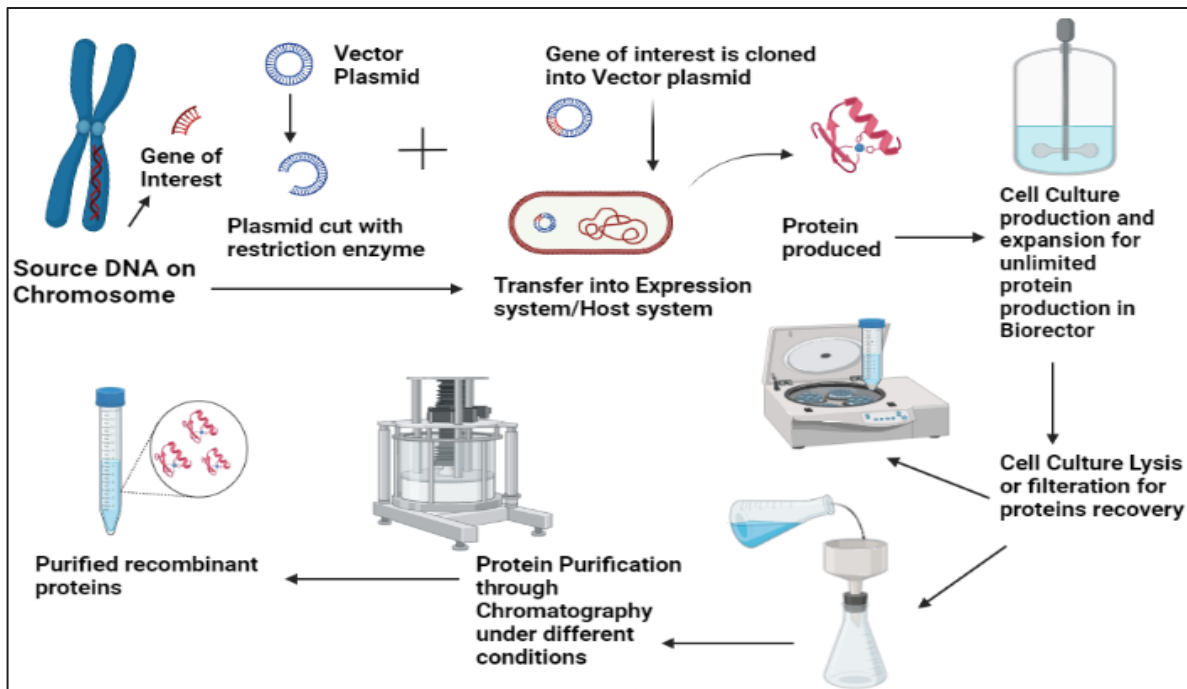


Fig. 3. General methodology for recombinant protein production in expression system and afterward purification

expression system because of certain key factors. The selection of a prokaryotic bacterial expression system is considered an attractive choice because of its low expense cost, medium productivity, and rapid growth of bacteria [17]. Most widely used bacterial expression system has been described as follows:

3.1 *Escherichia coli*

The most widely used bacteria for recombinant protein production is the gram-negative rod, *E. coli* because of its certain significant characteristics i.e., its short replication time and extremely fast growth kinetics enable fast achievement of high cell densities. The reagents and culture media required to grow *E. coli* are quite simple and inexpensive. The genetic manipulation and transformation of *E. coli* are comparatively simple and straightforward due to its genetics, biochemistry, and metabolism [17, 18]. It is comparatively easy to manipulate the genetic information of *E. coli* as compared to other bacterial expression systems such as *Streptomyces* and *Pseudomonas* system [17]. Besides this, therapeutic protein production in *E. coli* eliminates the requirement of optimization step as various standard plasmids can be employed easily. Usually, *E. coli* is used with T7 bacteriophage RNA polymerase. Moreover, it is now considered the

most appropriate approach to start the recombinant protein production process [9]. In 1982, the first biopharmaceutical product, approved by Food and Drug Administration (FDA) was biosynthetic insulin, engineered in an *E. coli* expression system [19]. Now a days, the *E. coli* expression system is widely used for several other therapeutic product productions such as tumor necrosis factor, human growth hormone, interleukins etc. [20, 21]. *E. coli* with a T7 RNAPol expression system is best suitable for the production of non-glycosylated proteins. *E. coli* is still under research to gain more and more understanding regarding its central dogma and post-translation modifications. *E. coli* is the most suitable expression system because it can tolerate a range of environmental conditions and can store the recombinant proteins in almost 80 % of its dry mass [1]. Figure 4 illustrates the possible strain improvement strategies for *E. coli*.

3.2 Lactic Acid Bacteria

Lactic acid bacteria (LAB), the gram-positive non-sporulating anaerobic rods, have long been used in multiple dairy fermentation processes as recombinant microbial cell factories. Among the other genera of LAB, *Lactococcus* and *Lactobacillus* are most commonly employed either as cell vectors to deliver therapeutic molecules or

Vector	<ul style="list-style-type: none"> • Vector change helps protein solubilization in <i>E. coli</i> host
Host strain	<ul style="list-style-type: none"> • Use of genetically altered <i>E. coli</i> strains based on protein suitability for better results
Culture parameters	<ul style="list-style-type: none"> • Changes in <i>E. coli</i> culture conditions will increase gene expression and protein solubility
Co-expression	<ul style="list-style-type: none"> • Co-expression of protein of interest and its important counter-part gene for recombinant proteins production and stability
Gene sequence	<ul style="list-style-type: none"> • Changes in gene sequence without any change in protein's functional domain brings stability to recombinant protein and increases its expression

Fig. 4. Strain improvement strategies for *E. coli*.

as producer cells [20]. *Lactococcus lactis* is one of the most promising LAB for recombinant protein production. Different *Lactobacillus* strains such as *L. reuteri*, *L. gasseri*, and *L. Plantram* are used for the production of green fluorescent proteins, and enzymes such as beta-glucuronidase, beta-galactosidase, and aminopeptidase. Being safe and non-pathogenic, LABs have been declared a Generally Recognized as Safe (GRAS) organism by the food and Drug Administration (FDA) [22]. LABs do not contain exotoxins in their cell membrane hence providing an endo-toxin-free expression system. Other key characteristics of LAB that makes them attractive expression systems include easily scale-able, cheap, safe for food, and production of heterologous membrane proteins. Novel genetic manipulation techniques such as CRISPR-Cas9 has also been used to transform LAB for the production of several therapeutic proteins [23]. LAB has a potential economic impact on the fermentation industry because it is used as an expression system for the production of lactic acid, milk products, wine, meat, high-grade metabolites, and antimicrobial peptides [24].

3.3 *Pseudoalteromonas haloplanktis*

One of the fastest growing and eligible expression system psychrophile is *P. haloplanktis* TAC125 which was isolated from a seawater sample from

an Antarctic coast. It is characterized as gram-negative bacteria which has the ability to grow at low temperatures (0–30 °C). The eligibility of *P. haloplanktis* for an expression system has also been improved by certain genetic modifications [10]. Antibody fragment production has been reported by using a cold-adapted platform instead of the conventional mesophilic platform which usually uses *E. coli* [25, 26]. Such platforms have also proved very beneficial for the production of some delicate and heat-sensitive proteins such as alpha-glucosidase and Human Nerve Growth Factor h-NGF [10, 27]. The production of h-NGF in *E. coli* failed as the h-NGF accumulated in inclusion bodies instead of folding accurately. In contrast, h-NGF folded in a proper dimeric form remained soluble and translocated in periplasm when expressed in *P. haloplanktis* TAC125 [10]. Similarly, alpha-glucosidase, a recombinant enzyme of *S. cerevisiae* was recovered efficiently in a soluble and active form from *P. haloplanktis* TAC125 as compared to that of *E. coli* in which alpha-glucosidase became insoluble [27].

3.4 *Pseudomonas*

Pseudomonas can grow rapidly and secrete protein efficiently. Different *Pseudomonas* species i.e., *P. aeruginosa*, *P. fluorescens*, and *P. putida* have shown the best protein yield and efficient expression

as compared to *E. coli* and hence can be used as the best alternative to *E. coli* for certain proteins [9].

3.4.1 *Pseudomonas putida*

Pseudomonas putida (strain KT2440) is a gram-negative soil bacterium which is widely used in a cell factory to produce industrially important proteins because of its extraordinary features. These features include versatile metabolism, rapid growth, minimal nutrient requirements, and tolerance to stress conditions. The extensive biochemical network of *P. putida* facilitates extensive production of NADPH instead of ATP. This property enables it to tolerate stress conditions [28].

3.4.2 *Pseudomonas fluorescens*

A proprietary expression system of *P. fluorescens* has been developed for the efficient production of recombinant proteins. This system is more advantageous as compared to conventional *E. coli*. Protein production in *P. fluorescens* can be carried out even in depleted oxygen concentration and it also prevents the accumulation of acetate in the expression system. However, it shares some characteristics with *E. coli* such as growing ability in saturated cell density (N100 g/L) and over-expression of proteins i.e., half of the total protein [136]. *P. fluorescens* fermentation does need strict control of glucose concentration and aeration passage. The highest production rate of nitrilase, a recombinant enzyme, has been reported as 25 g L⁻¹ using *P. fluorescens* as an expression system. Moreover, the *P. fluorescens* expression system proved advantageous for an insecticidal protein with a yield of 3–4 g L⁻¹ which is comparatively very high as compared to that of *E. coli* i.e., 100 mg L⁻¹ [9, 29].

3.5 Other bacteria

With the advancement in research and technology, many other bacterial expression systems have emerged as successful alternatives. One of the most noteworthy bacterial systems is the gram-positive bacteria *Streptomyces* which has an efficient secretory system. It secretes a high concentration of the desired proteins in the medium which reduces some recovery steps [9]. Another choice of expression system is *Ralstonia*.

4. FUNGI

Filamentous fungi have been used as commercial organisms to produce pharmaceutical and enzymatic products. The versatile metabolic ability of fungi makes it an attractive and outstanding cell factory. Fungi also have the ability to express several prokaryotic as well as lower eukaryotic genes after genetic manipulation. Filamentous fungi have a strong capacity for secretion due to which they are also considered one of the most promising expression systems for recombinant protein production. However, there are a few fungal species (*Aspergillus* sp. and *Trichoderma* sp.) that are used to produce a competitive level of recombinant protein at an industrial scale [27].

4.1 *Aspergillus*

Aspergillus is one of the most extensively studied genera from a research perspective. Its species are considered model organisms i.e., *A. nidulans*. Other species i.e., *A. oryzae* and *A. niger* have great importance in citric acid production even at the industrial scale [33]. Several molecular genome editing tools such as RNA interference-RNAi, selection markers, and CRISPR/Cas9 are also being used to manipulate the *Aspergillus* sp. genome to get the desired level of protein of interest [34, 35]. *A. niger* genome has been successfully edited using CRISPR/Cas9 which incorporate double-strand breaks in the DNA sequence. Moreover, this technology is also being used for other *Aspergillus* sp. Being considered as GRAS, the genetically modified *A. niger* has been employed at an industrial scale to produce very significant proteins i.e., human lactoferrin, calf chymosin, and neoculin, a plant-derived sweetener [36-42]. The yield of all these proteins have been increased by optimizing the physiological growth conditions of the fungi.

4.2 *Trichoderma reesei*

Another important fungus is *T. reesei* which also contains an extraordinary secretory system. Its genome can be modified by aggressive mutations to produce extracellular cellulase. A yield of 100 g/L cellulase production was reported by *T. reesei* of which 60 % was characterized as Cel7a (CBHI) while 20 % was Cel 6a (CBHII) [43]. Initially, *T. reesei* was exploited for the production

of calf chymosin [44, 45] later on it was reported to efficiently produce antibody fragments [46]. The higher extent of recombinant protein production was attributed to the taxonomic relatedness of the gene of interest and the host. *Trichoderma* is used to produce recombinant cellulase by incorporating its genes into cassettes [43, 47]. Usually, Cel 7a (CBHI) single peptide is responsible to mediate the high secretion level of this protein [48].

4.3 Other fungi

There is comparatively less information regarding the gene sequencing of filamentous fungi other than *Aspergillus* and *Trichoderma* specie. However, Pectinases, hemicellulases, and cellulases are reported to be produced by several *Penicillium* species such as *P. emersonii*, *P. funiculosum*, and *P. purpurogenum* respectively [43]. Another fungal system (*N. crassa*), reported to grow at a maximum rate in normal media, has been genetically and biochemically characterized. It has the potential to produce and secrete proteins at a higher level. Its genome size is 40Mb, of which there are 10,000 protein-coding genes. Most of these genes are responsible for secondary metabolite production in *Neurospora* [43, 49]. Now a days, *N. crassa* has been adopted as an expression system to produce vaccine subunits such as neuraminidase antigens (NA) and influenza hemagglutinin (HA) [50]. Recently, many fungal strains i.e., *T. reesei*, *N. crassa*, and *Aspergillus* species are being used in the production of antibodies [51-53].

5. YEAST

Yeast, unicellular microorganisms having the ability to process proteins like eukaryotes i.e., assembly, folding, and post-translational modifications, has always remained an organism of interest to be used as an expression system. Due to easy manipulation in genes and efficient growth rates, yeast cells are beneficial hosts. Moreover, it does not possess any oncogenes or endotoxins. *Saccharomyces cerevisiae* has been used to express the majority of recombinant proteins since 1980 [61]. Food and Drug Administration (FDA) has declared *S. cerevisiae* as GRAS (generally regarded as safe). But there is one problem and that is yeast is not good for large-scale productions because it requires efficient machinery for its fermentation. Moreover, products produced by *S. cerevisiae* mostly remain

in periplasmic spaces due to hyperglycosylation [62, 63]. These products start degrading after some time and it is very hectic to remove them from our desired product. These drawbacks led to the quest for new species of yeasts for another expression system, most of these efforts were made by using nonconventional Yeasts such as *Pichia pastoris*, *Hansenula polymorpha*, *Sarcoscypha occidentalis*, *Pichia methanolica*, *Zygosaccharomyces rouxii*, *Candida boidinii*, and *Kluyveromyces lactis*, etc. [55, 64-66].

5.1 *Saccharomyces cerevisiae*

For almost the last thirty years, *S. cerevisiae*, a eukaryotic microorganism, has been used to express different recombinant proteins [67]. *S. cerevisiae* was used to express the recombinant form of the first vaccine i.e., Hepatitis B and it was produced intracellularly. *S. cerevisiae* also comes in the first row when approval for any recombinant therapeutics is required from FDA and EMEA, recombinant therapeutics produced from it always get green signals from these agencies [68]. Hirudin, Platelet-derived growth factors, Hepa-B surface antigen, insulin, and GM-CSF (Granulocyte macrophage-colony-stimulating factors) are some of the main products of *S. cerevisiae* which are currently available in the market for use [6].

5.2 *Pichia pastoris*

P. pastoris is a methylphilic yeast because it has the ability to use methanol to fulfill its requirement of carbon in the absence of any other carbon alternative [69]. *P. pastoris* has a strong tendency to secrete protein even if they have a high molecular weight. This characteristic makes them better than *S. cerevisiae* in which heavy proteins retain in the periplasm. Moreover, it is comparatively easy to purify the secreted proteins from the extracellular medium. The strains of *P. pastoris* i.e., protease deficient strains (SMD1163, SMD1165, SMD1168) and Auxotrophic mutant (GS115) are commonly used and are derived from wild type NRRL-Y 11430 strain. *P. pastoris* strains have also been characterized on the basis of their ability to utilize methanol i.e., Mut+, MutS, and Mut- [70]. A therapeutic polypeptide of 60 amino acids was produced by *P. pastoris*. It was further approved as safe by Food and Drug Administration FDA in 2009 to treat hereditary angioedema. A Comparison

of the yield of some recombinant protein products produced by bacteria and other expression systems is given in Table 1.

5.3 *Kluyveromyces lactis*

Since the 1950s, the production of β -galactosidase (also known as lactase), as well as the heterologous appearance of rennin (bovine chymosin) is done by using *K. lactis* [9]. Many features of *K. lactis* played their role in its popularity for the production of r-protein. Some of these features are as follows, LAC4 which plays the role of such a powerful inducible promoter. This promoter is regulated by even low amounts of glucose. It also has the ability to use whey as well as lactose-like cheap substrates. For its approval as a GRAS strain and its ability to produce proteins with high molecular weights [39], a complete sequence of its genome [67], as well as a kit for protein expression is commercially available from NEB [47]. Previous literature about *K. lactis* is mostly about the MAT α CBS 2359 mating strain and GG799 haploid strain which is a wild-type strain that is known as a good host as it is added in the commercially available kit.

5.4 *Yarrowia lipolytica*

For expressing the heterologous proteins, the use of *Y. lipolytica* expression system is getting popularity due to the following reasons; i) a large amount of proteins with high molecular weight is secreted

due to its inherent ability; ii) pathway named as co-translational translocation is analogous to eukaryotes and could be the reason for the secretion of proteins [76]. This system of protein secretion is exactly opposite to the pathway named as post-translational translocation and is mostly present in *S. cerevisiae*; iii) sugar is not fermented by this organism, iv) completely sequenced genome is available [69, 77]; v) fermentation with high density is possible; and vi) many GRAS processes on the industrial level are approved by FDA by using this organism [69].

5.5 Other yeast

Hansenula polymorpha expression systems which include a hepatitis B recombinant vaccine, insulin, phytase, hirudin, and alpha 2a interferon, along with food supplements known as lipase as well as hexose oxidase having GRAS notification by FDA. A dimorphic yeast named as *Arxula adenivorans* is a temperature-dependent yeast. It has the ability to grow as budding cells as well as mycelium. *A. adenivorans* also has the ability for secreting extracellular enzymes during cultivation in the surrounding medium. It mostly secretes the proteases, glucosidases which include xylosidase, cellobiose, pectinases, invertase, acid phosphatases, glucoamylase, trehalose, phytase, and β -glucosidases. It also releases RNase [69]. A fission yeast named as *Schizosaccharomyces pombe* have the ability to grow as preferential

Table 1. Comparison of the yield of some recombinant protein products produced using bacteria and yeast expression systems

Recombinant protein	Example of Expression-system used	Highest product yield (g/Lh)	Reference
Interleukin-6	<i>E. coli</i>	7.5mg mL ⁻¹	[131]
	<i>P. pastoris</i>	0.28 mg mL ⁻¹	[71]
Riboflavin	<i>C. famata</i>	0.11 g L ⁻¹	[72]
	<i>B. subtilis</i>	0.33 g L ⁻¹	[1]
	<i>A. gossypii</i>	0.07 g L ⁻¹	[132]
	<i>C. ammoniagenes</i>	0.21 g L ⁻¹	[54]
Glutamic acid decarboxylase	<i>S. cerevisiae</i>	0.46 mg mL ⁻¹	[131, 73]
	<i>E.coli</i>	12.5 mg mL ⁻¹	[71]
	<i>P. pastoris</i>	0.42 mg mL ⁻¹	[73]
	<i>Spodoptera frugiperda</i> cells	0.02 mg mL ⁻¹	[71]
Insulin	<i>S. cerevisiae</i>	0.075 g L ⁻¹	[71]
	<i>E. coli</i>	4.34 g L ⁻¹	[1]
	<i>B. subtilis</i>	1 g L ⁻¹	[74]
	<i>P. pastoris</i>	3.07 g L ⁻¹	[75]

haploids. Having a complete genome sequenced eukaryotic list it is numbered at 6 [78]. Having a complete availability of proteome in Swiss Prot as well as UniProtKB it is numbered at 3 in the eukaryotic list following *Homo sapiens* and then *S. cerevisiae*. For expressing the mammalian proteins *S. pombe* is considered an eye-catching host and a great area of research [79, 80]. A brief list of microorganisms that have applications in recombinant protein production are listed in Table 2.

6. PLANTS

With the discovery of growth hormones in tobacco plants the production of recombinant proteins by using plant expression machinery came into existence. In today's world, three types of methods are used for the production of recombinant proteins: by forming transgenic plants, by using systems relying upon plant-tissue, and by using cell cultures. Bacterial infection and viral infection are used for the transformation methodology. Some direct methods such as biolistic bombardment and PEG-mediated technique can also be used for transformation methodology [17].

Therapeutic recombinant protein production is done by using plant expression systems and the main focus is to enhance the quantity and efficiency of produced recombinant proteins [81, 82]. The

main features of plants for their use as recombinant proteins producer are as follows: growth conditions are cheap, the manufacturing procedures are well understood, scalability levels are very high, their high ability for the production of complex proteins, the infrastructure of the already existing industry, the ability for the rapid production, less chances of contamination with human pathogens [83].

By using plant sources for the production of recombinant biopharmaceuticals the production can be increased and costs can be decreased. The plant factories producing recombinant proteins possess the following qualities mentioned in the literature: safety, insensitivity to changes in temperature as well as pH, low cost, metabolites presence, high stability, easy and cheap storage of produced drugs, and ability to produce proteins named as N-glycosylated [130]. The most important feature of using transgenic plants is that high production is maintained by investing low costs while compared with other expressions of prokaryotic as well as eukaryotic systems it costs lower up to 50 percent [84, 130]. Hypothetically arguing the plant fruits, seeds and leaves might be a rich source of therapeutic proteins. The transgenic plants used for the production of recombinant proteins show an expression ranging from 0.001 % to 46.1 % [85]. The ability of transgenic plants to store recombinant proteins in cell compartments as well as the plant organs makes them different from the other plant

Table 2. Bacterial, fungal, and yeast strains used in recombinant proteins production

Expression systems	Strains used	Reference
<i>E.coli</i>	<i>E.coli ArcticExpress</i> , <i>E.coli BL21</i> , <i>E.coli BL21-Codonplus (RIL)</i> , <i>E.coli M15</i> , <i>E.coli Lemo21(DE3)</i> , <i>E.coli C43(DE3)</i>	[22]
<i>Yeast</i>	<i>Z. bailii</i> , <i>C. famata</i> , <i>H. polymorpha</i> , <i>P. methanolica</i> , <i>P. pastoris</i> , <i>P. stipites</i> , <i>K. lactis</i> , <i>Z. rouxii</i> , <i>S. occidentalis</i> , <i>S. cerevisiae</i> , <i>Y. lipolytica</i> , <i>C. boidinii</i>	[54, 55]
<i>Aspergillus</i> <i>Bacillus</i>	<i>A. awamori</i> , <i>A. terreus</i> , <i>A. niger</i> , <i>A. sojae</i> , <i>A. nidulans</i> , <i>A. oryzae</i> <i>B. subtilis KL03</i> , <i>B. subtilis 168</i> , <i>B. megaterium MS941</i> , <i>B. licheniformis BL10GS</i> , <i>B. subtilis 1A751P7</i> , <i>B. subtilis IH6622</i> , <i>B. subtilis BNA</i> , <i>B. brevis</i> , <i>B. licheniformis</i> .	[43, 53, 56, 57] [1, 58]
<i>Pseudomonas</i>	<i>P. fluorescens</i> , <i>P. putida</i> , <i>P. aeruginosa</i>	[1, 9]
<i>Streptomyces</i> <i>Corynebacterium</i> <i>Trichoderma</i>	<i>Streptomyces lividans</i> <i>C. glutamicum</i> , <i>C. ammoniagenes</i> , <i>T. reesei</i> , <i>T. altroviride</i> and <i>T. virous</i>	[1, 22, 9] [9] [43, 59]
<i>Rhizopus</i> <i>Fusarium</i> <i>Ralstonia</i>	<i>Rhizopus oryzae</i> <i>F. graminearum</i> <i>Ralstonia eutropha</i>	[43] [43, 60] [1]

systems [86, 87].

6.1 Production of Recombinant Proteins by using Plant Species

6.1.1 Tobacco

Recombinant protein production on the laboratory level is mainly done by using Tobacco plants. The Tobacco plant plays a very important role in the mass production of recombinant proteins. The yield with Tobacco plants is “more than 100,000 kilograms/hectare, especially for the close-cropped tobacco” [88]. It also has the ability for the fast scaling-up process as it can produce large amounts of seeds. Some considerations to be kept in mind while looking for tobacco plants are as follows: protein storage in the aerial parts of the plants especially leaves is unstable which may cause product degradation. To avoid this degradation, product should be extracted as soon as they are expressed, alternatively the leaves can be dried or frozen for product extraction. Due to the presence of alkaloids which are toxic in nature and phenols the tobacco plant can also be used for the downstream process.

6.1.2 Cereals

Cereal seeds act as marvelous storage devices for proteins as they are outfitted with storage vesicles for protein storage. They are also adhering to an intracellular environment with drying conditions which helps them to reduce the activity of protease, and also helps in reducing the hydrolysis by non-enzymatic sources. Among all the food crops, the highest biomass yield is recorded for Maize [89, 90]. Now a days, Maize has been used for the biomass production of some recombinant antibodies, avidin as well as trypsin [82, 88]. Dry cereals such as wheat and rice seeds show high stability for recombinant protein storage and that is why they can be stored at normal temperatures without a noticeable loss in their activity. Due to the self-fertilizing ability of rice, the risk of transfer of transgenes to other plants have been reduced [82, 91].

6.1.3 Legumes

The worth mentioning legumes are alfalfa, soybean, and pea which have been used for the recombinant production of therapeutic proteins. Atmospheric

nitrogen is fixed by the legume plants due to which they don't need the nitrogen in their fertilizers and thus leads to the low cultivation cost. The biomass yield of leaves is lower than that of Tobacco plants. Peas are being used as the expression systems due to the high content of proteins in their seeds [88].

6.1.4 Fruits and vegetables

Recombinant proteins especially therapeutic proteins are produced by using the vegetables and fruit crops such as tomato, lettuce, and most importantly potato. One of the main features of this system is that the produced proteins could be used for oral consumption after minimal processing. Conversely, quality maintenance and dose-ranging are still the main challenges to be addressed yet [88, 92, 93].

7. ANIMALS

Transgenic animals have been developed from cows, mice, sheep, goats, and rabbits for recombinant protein production as shown in Figure 5. Aquatic animals are also being explored for the same reason. Human factor IX and AAT (α antitrypsin protein) are being produced by the transgenic sheep in milk as shown in Figure 6. β -lactoglobulin and tPA by transgenic mice [94]. The amount of recombinant proteins produced in animal milk is as follows: anti-thrombin III is being produced in goat milk around 14 g/L, α -1-antitrypsin is being produced in sheep milk around 35 g/L and α -glucosidase is being produced in rabbit milk 8 g/L. For recombinant proteins production genes are usually taken from human sources. The expression of non-milk foreign proteins is very low than milk proteins [95].

Transgenic mice urine is the main source for the production of growth hormones for humans. The amount produced by the mice is about 0.1 to 0.5 mg/L [1]. One of the most advent features of using the urinary system for recombinant protein production is that the animals start to urinate at an early age rather they lactate at a specific time in their life. About 10,000 L of milk is produced by a cow after hormonal treatment as compared to the amount of urine which is 6000 L. The main disadvantage of recombinant protein production by transgenic animals is the amount of time required for the estimation of production level. This time is

almost 32 months in the case of cows, in mice it is 3.5 months, in sheep it is 28 months and in pigs, it is 15 months [96]. For a cow to keep up, we need almost \$ 10,000 per year.

Previously, transgenic animals were being used for the production of lymphokines and vaccines, but now scientist have shifted their focus towards protozoa and are exploring its potential to produce important protein products. Transgenic trypanosome is far better than transgenic animals for the production of heterologous proteins [1]. These benefits include i) with the help of homologous recombination, precise and stable integration is done, ii) the integration can be done on many sites due to which the expression from the multiple unit complexes can come out, iii) high densities growth and easy cells maintenance in the semi-defined medium.

8. INSECTS

An IC (insect cell) system is working as a solution between the two main expression systems which are the mammalian system and the bacterial system. With the development of BEVS (baculovirus expression vector system) recombinant protein production is welcomed by the IC platform [15]. The development of the IC platform includes two steps; first is the multiplication at the desired concentration and the second step is the addition of baculoviruses for infection purposes, this baculovirus also contains GOI [97]. The origin of insect cells used for this purpose came from *Drosophila melanogaster*, *Autographa californica*, and *Spodoptera frugiperda*. These cells are used as they are susceptible to baculovirus infection [97, 98]. Some other insects can also be used for the development of recombinant proteins such as tPA (plasminogen activator), hGAD65, parasitic proteins as well as viral proteins [17, 97, 98].

8.1 Silkworm

Bombyx mori, a silkworm, produces large quantities of silk proteins in its silk glands which can be used to form cocoons. Silk proteins are the major components of silk and include sericin and fibroin proteins majorly [99]. In the case of transgenic silkworms, the recombinant proteins are expressed in the silk glands. The expression and place of

the recombinant proteins can be controlled by controlling the location of the genes that are able to produce the silk proteins. By using PGS (Pre-implantation genetic screening) we can localize the expression of silk proteins in the inner core of fibroin. By using MSG (Monosodium glutamate), the expression of silk proteins can be localized into the outer layer of sericin [99]. Table 3 represents the recombinant proteins obtained from different types of IC.

9. MICROALGAE

Microalgae, also known as photosynthetic microorganisms, are a diverse group of organisms capable of using sunlight to produce proteins, carbohydrates, and lipids [112]. They are usually considered as unicellular eukaryotes in spite of the fact that some cyanobacteria which are prokaryotic organisms also referred to as microalgae [113]. In recent years, interest has been developed in using microalgae for the production of biofuels as well as therapeutic proteins [1]. Some species of microalgae can be used as a substitute for fossil fuels in the biofuel industry due to their astonishing oil content [138]. Another reason for their biotechnical focus might be due to their ability to act as extraordinary bioreactors for the production of recombinant proteins on large scale. Microalgae exhibit the qualities of both prokaryotic and eukaryotic expression systems such as high progression rates as well as post-transcriptional and translational amendments. A major benefit over previously employed expression systems such as insect cells, mammalian cell lines, bacteria, and yeast is that algae have a phototropic lifestyle, making their cultivation CO₂-neutral and simply requiring relatively minimal expenditures [139]. Severe infections can be prevented by the use of vaccines but due to their costly production, the process of vaccination is highly influenced particularly in developing countries. Almost all the time, generation of antigen-grounded vaccines bearing heterologous expression is simpler than the antibody generation procedures. Despite this advantage, these antigen vaccines still required mammalian cell lines' expression system for their intricate post-translational modifications. In addition, they also face high risks of contamination by human pathogens. Microalgae have excellent opportunities in this regard as they do not serve as

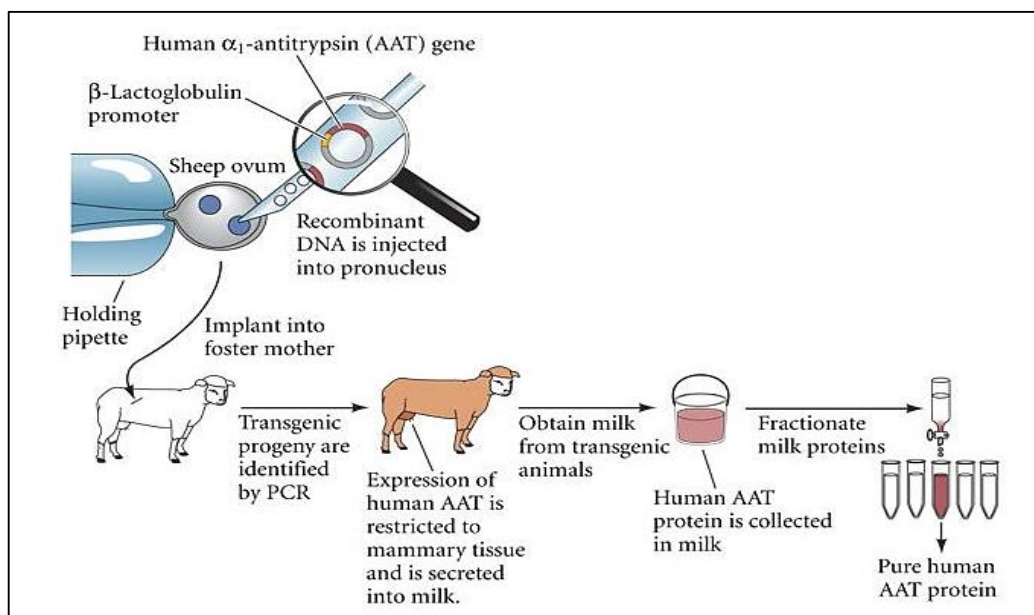


Fig. 5. Proteins pharmaceuticals production by the cloning of transgenic animals [138].

Table 3. List of recombinant proteins obtained from different IC (insect cells)

IC (insect cells) of	Recombinant proteins	References
<ul style="list-style-type: none"> • <i>Drosophila melanogaster</i> • <i>Autographa californica</i> • <i>Spodoptera frugiperda</i> 	<ul style="list-style-type: none"> • tpA(plasminogen activator) • hGAD65 • Parasitic proteins • Viral proteins 	[97, 98]
<ul style="list-style-type: none"> • Silkworm cocoons (Transgenic) 	<ul style="list-style-type: none"> • Human collagen α chain • Human-serum albumin • Monoclonal antibody (mouse) 	[99, 100, 101]

hosts for human infections and have rapid growth rates. In fact, once the proper bioreactors are in place, growing them is relatively inexpensive as they only require light and water [140].

9.1 *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii, is known as the model algae and is mostly used as a model for representing the eukaryotic micro-alga. Not only for its metabolic and genetic ability but also for its rapid reproduction. The sexual cycles are not only rapid but they are also controllable [107]. Microalgae as an expression system have expressed almost 20 recombinant proteins, especially in the *C. reinhardtii* [107]. *C. reinhardtii* is being used extensively because it is stable, and easily transformed especially for the transformation of mitochondrial organelles, nucleus, and chloroplast, quick reproduction of transformants, availability of

many tool kits at the molecular level, and alternative growth of organisms such as heterotrophic growth and phototrophic growth. 16-5 % is the rate for the expression level of recombinant proteins. Proteins developed in chloroplast have higher levels of expression than expressed in the nucleus. HSV8 is the first protein that could be expressed in this organism [1].

9.2 Other algae

Some other algae are also being explored for recombinant protein production. With time the development of transgenic algae is enhancing. *Charophyte* alga is used for expressing exogenous genes [114]. *Gonium pectoral* which is a volvocine alga has a nuclear genome and it is successfully transformed [115] as it has the *C. Haematococcus pluvialis* [116, 117] because it is co-cultivated with the help of agrobacterium. *Chlorarachniophyte*

Lotharella amoebiformis which is a marine organism is successfully put into the transient transformation [118]. Some other successful transformations are done by using the *Ulva Pertusa chlorophyta* alga [119]. *Cyanidioschyzon merolae* also known as the red alga is used for successful transformation [120]. *Dunaliella salina* which is a previously known alga is also being used for the expression of recombinant proteins [121]. The genetics of cyanobacteria has also been explored for this purpose.

10. MAMMALIAN CELL LINES IN USE FOR RECOMBINANT-PROTEIN PRODUCTION

Mammalian cell lines offer a range of cell lines made from different tissues and support various proteins' growth. These cell lines have all the needed machinery for protein expression and release. Among many reasons for them being a preferred expression system, one reason is their ability to synthesize proteins which is closely related to proteins present in humans in terms of their molecular structure and biochemical properties [122]. They are preferably selected when it comes to glycosylated protein production as these proteins are usually complex [123].

Most of the biopharmaceutical research uses two types of mouse cell lines i.e., myeloma cell lines (NS0) and hybridoma cell lines (SP2-0) as well as two types of hamster cell lines known as CHO cell lines (Chinese hamster cell ovary cells 1) and BHK cell lines (Baby hamster kidney cells) [122]. The major reason why these cell lines are different from other expression systems is due to their ability of N- and O-linked glycosylation as proteins that work in glycosylation encodes 2 % of the human genome [124]. Nearly all mammalian cells have the necessary machinery for recombinant protein production, but only a few meet the criteria of bioreactors such as mouse myeloma cells NS0, CHO, Sp2/0, and BHK. Viral vectors which are helpful for gene therapy are usually formed by using mammalian cell lines. Many vaccines are manufactured by the use of mammalian cell lines such as rabies, measles, rubella, and hepatitis A [141]. FDA has approved 27 biopharmaceutical products and 12 of which are being produced in mammalian cells [123].

10.1 Human cell-lines

In the quest to produce human recombinant proteins, the current major focus for biopharmaceutical industries is to look for an expression system that is not only safe clinically but can also give a high yield of proteins [133]. As human cell lines have glycosylation machinery, a few powerful human lines have emerged as a substitute for human recombinant protein production on a commercial level [108]. HEK293 is the human cell line currently used for the production of different therapeutic products such as human-cl, rFVIII_{FC}, drotrecogen alfa, etc. Another human cell line HT-1080 also used for the production of pharmaceuticals such as Epoetin delta, Agalsidase alfa, and Idursulfase [141]. Table 4 represents the recombinant proteins produced by different expression systems. Further, Table 5 shows the main advantages and disadvantages of each type of expression system used.

11. CONCLUSION AND FUTURE PERSPECTIVE

A huge extent of therapeutic protein marketing comprises of a variety of products such as antibodies, vaccine subunits, hormones, and enzymes. In order to meet the day-by-day increasing need for these therapeutic proteins, DNA recombination technology is being used. All recombinant proteins are naturally different from each other and various challenges are kept into consideration while choosing an expression system for their production. Therefore, it is very significant to investigate the potential and limitations of several expression systems to choose the suitable one for particular protein production at an industrial scale. The optimization criteria of an expression system is evaluated on several factors such as productivity, efficiency, physiological characteristics, total cost, safety, convenience, and down-streaming conditions. There are certain challenges which are associated with recombinant protein production at a higher scale i. e., maintaining protein production in higher cell densities, separation of cell debris from viable cells and product of interest, separation of yield without causing cell lysis, downstream processing and mass transferring of the products [134]. DNA recombinant technology can also produce recombinant proteins comparatively

Table 4. List of recombinant proteins produced in different expression systems

Expression system type	Recombinant protein	Application	Reference	
Insects (Transgenic silkworm cocoon)	Human collagen α chain	Biomaterial, DDS, therapeutics	[100]	
	Human-serum albumin	Therapeutics	[101]	
	Monoclonal antibody (mouse)	Therapeutics, diagnostics	[99]	
Bacteria	Lactic acid bacteria (LAB)	Gamma-aminobutyric acid (GABA)	Anti-hypertensive & antidepressant activities	[102]
	<i>P. fluorescens</i>	Granulocyte colonystimulating factor (G-CSF)	Drug candidate	[103]
Plants	<i>N. benthamiana</i>	Protein E envelop from Zika virus	Zika virus	[104]
	Barley seed (<i>Hordeum vulgare</i>)	Human Epidermal Growth factor	Burns treatment	[17]
	<i>Arabidopsis thaliana</i>	Recombinant human intrinsic factor	Vitamin B12 deficiency	[84]
Algae		High mobility group protein B1	Therapeutics	[105]
		cholera toxin B subunit (CTB-D2)	Oral vaccine	[106]
		Human metallothioneine-2 (hMT-2)	Pharmaceutical, UV protection	[107]
Human Cell line	Hek293	Proteglycan	[108, 109]	
	Hek293EBNA1	Erythropoietin	[110]	
	PERC.C6	IgM	[111]	

better than the native ones by manipulating the protein sequence at the genetic level. Therefore, recombinant proteins can be adopted in several applications such as therapeutics, diagnostics, health maintenance, etc. With technological advancement, nanotechnology has also been adopted with recombinant DNA technology for the production of more advantageous and efficient recombinant proteins [135].

12. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Table 5. Comparison of advantages and disadvantages of using various expression systems

Expression system	Advantages	Disadvantages & Challenges	Ref.
Bacteria	<p>Simple and low price media, High cell density, Easy to cultivate, Rapid growth rate, Cost-effective, Virus free, Optimized growth procedures, Genetically characterized, Fermentation processes worked out for scale-up, FDA approved SOPs</p>	<p>Post-translational modifications don't occur here, Secreted proteins are of low level, Inclusion bodies formation from protein aggregate, Endotoxin production, Susceptible to proteases degradation, Codon usage is preferential, Chances of misfolding and export issues for proteins larger than (>30kDa)</p>	<p>[1, 71, 76, 82, 125, 126]</p>
Yeast	<p>High cell density, Highly adaptable to fermentation processes, Genetically characterized, No endotoxin production, High-level protein secretion, High yield, Durability, Cost-effective, Maximum yield in chemically defined media, Product processing similar to mammalian cells, Can handle S-S rich proteins, Can assist in protein folding, Can glycosylate proteins, Correct folding in functional recombinant proteins, Low-budget purification processes, FDA approved SOPs</p>	<p>N-or O-linked glycosylation pattern is different from a eukaryote, Hypermannosylation, Proteolytic degradation, Hyperglycosylation, Produced glycoproteins are not found fit for human consumption</p>	<p>[1, 71, 76, 126, 127]</p>

Expression system	Advantages	Disadvantages & Challenges	Ref.
Fungi	<p>Poor growth conditions, Fast growth, High density, Well-characterized, Optimized growth procedures, Scalable, Moderately amenable to genetic engineering, Correct protein folding and processing, Less time consumption for production, Production strains are stable, Existing regulatory bodies' approval</p>	<p>Immunogenic sugars with nonhuman glycosylation</p>	[82]
Insect (Baculovirus)	<p>Post-translational modification is similar to higher eukaryotes, Proper protein folding, Able to produce glycosylated recombinant proteins, Produced protein is of high level, Up to 15 kb insert expressed, Noninfectious, and safe, Easy scale-up, Flexibility in protein-size, Signal peptides cleavage is efficient, and can express multiple genes at a time, FDA-approved SOPs</p>	<p>Slow growth rate, Expensive media, Multi-parallel protein expression is time-consuming and laborious, At late stages protein folding is improper, Stability of recombinant virus, Complex glycosylation not possible, Presence of lipidic envelopes in virions, Polyproteins processing is less efficient, Immunogenic sugars with nonhuman glycosylation, Undesired post-translational modifications</p>	[1, 76, 82, 126]
Plants	<p>Rapid growth, Low-cost purification, Suitable for glycosylated proteins production, Modification of any type is possible, Maximum and cheap scale-up possibility, Low growth costs, Can produce complex proteins, Relatively simple and cheap,</p>	<p>Highly specific to the plant of choice, Universal recombinant production system is not reported yet, Immunogenic sugars with nonhuman glycosylation, Lacks regulatory approval</p>	[1, 82, 126, 128]

Expression system	Advantages	Disadvantages & Challenges	Ref.
Animals	<p>Human pathogen contamination risk is low, Growth procedures are optimized, Protein complexes (Proper folding and assembly)</p> <p>Scaling up potential is massive, Post-translational modifications are correct, Harvesting is easy, Farming techniques are optimized, Cell lines are stable, Production cost is low, Existing regulatory approval</p>	<p>Costly, Transgenic organisms are difficult to create, Production time period is long, and Ethical as well as regulatory issues, Recombinant protein production system is poorly characterized, Low control,</p>	<p>[1, 82]</p>
Mammalian Cell lines	<p>Post-translational-modifications are very much similar to native protein, Safe, No immunogenicity concerns, Highly adaptive, Properly folding, Can produce glycosylated protein, High yields, Many current products give precedent to regulatory bodies, Active research and industry funding, FDA approved SOPs</p>	<p>Grow very slowly, Difficult to cultivate, Time-consuming, Large-scale culture limitations, Protein yields are low, Nutrient requirements are complex, Fermentation cost is very high, Viral and human pathogen contamination risks are very high, Expensive, Costs increase due to complex growth requirements, Complex cells create problems in cell lines engineering and understanding, Product is heterologous, Cell lines are unstable, Scaling-up is difficult</p>	<p>[1, 71, 76, 126, 129]</p>

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Impact of Climate Change on Marine Biodiversity: Current Challenges and Future Perspectives

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Abstract: Marine ecosystems have been affected by natural and human-induced pollution. In recent decades, land and sea levels have risen due to increases in temperature and CO₂ concentrations, as well as the accumulation of other toxic chemicals. The distribution and diversity of marine species have been affected by acidification, eutrophication, and anaerobic conditions, inhibiting the interaction process and increasing coral mortality and bleaching. Both cold- and warm-water coral reefs serve as food, shelter, and habitat for many important plant and animal species that have been severely impacted by ocean warming. Some marine plants, including mangroves and seagrasses, have been severely affected by ocean warming. However, the response of marine genotypes, i.e., mangroves, seagrasses, fishes, mammals, turtles, etc., varies depending on the type of environmental extremes and the nature of climate change. The species with lower immunity are vulnerable to ocean warming. Many researchers have studied that both marine flora and fauna are in danger if proper management and strategies are not developed. Here we discussed the negative impacts of climate change on marine ecosystems and also highlighted the new methods being used to protect marine species. In addition, various protocols developed to protect marine biota in the context of rising temperatures are briefly discussed along with future guidelines for marine ecosystem security. We suggest that marine biodiversity can be conserved through the establishment of marine protected areas and that novel epigenetic studies are needed to improve the genetic protection of marine species under abiotic stress and to minimize the risks associated with various anthropogenic activities.

Keywords: Acidification, Climate change, Epigenetics, Marine Ecosystem, Management and Policies, Ocean Warming

1. INTRODUCTION

The area extending 200 m below sea level is called the deep-sea region and is one of the most significant areas for countless important living organisms [1]. About 71 % of the total Earth's surface is covered by the ocean which is one of the

most important factors in controlling climate [2]. Fundamental primary compounds and other carbon and nutrients are formed in this area. For this reason, it is a suitable habitat for many seabirds, plants, fish, and other marine species. The warmth in this region is directly related to greenhouse gases [3]. Due to some anthropogenic activities and other

environmental changes, the marine ecosystem is harshly affected. Both direct and indirect human activities affect marine ecosystems. Similarly, natural and anthropogenic factors are responsible for environmental degradation. Sometimes the climate is affected by natural forces like volcanic eruptions, thunderstorms, and many others. A large part of the deep sea is affected by these harmful factors [3]. The increased contamination continues to cause degradation with negative impacts on food security, food safety, and marine biodiversity [4].

The negative impacts of human activities on the ocean ecosystem are increasing day by day [2]. Some of these changes have occurred rapidly in the oceans in recent decades [5]. Measuring these impacts on marine ecosystems is difficult because of their complexity and the diversity of many species [6]. Over the past 30 years, world temperature has increased by up to 0.2 °C/decade due to toxic greenhouse gases [7]. By 2015, world temperature has increased by about 1 °C due to high concentrations of toxic greenhouse gases (GHGs). The additional energy has been absorbed by the oceans. In addition, the heat of the uppermost layer of the Globe Ocean has increased by up to 14×10^{22} J since 1975, which corresponds to an increase in the temperature of the uppermost layer of 0.6 °C [2]. A further increase in Earth's temperature is estimated if 130-160 Pg of permafrost carbon is released into the air by 2100. Ocean pH is slowly decreasing at a rate of 0.02/decade over the past 30 years [8]. The temperature rise is affecting the terrestrial ecosystem and melting excess ice that is directly entering the water bodies, increasing the volume and stratification of the oceans [9]. The production of a large amount of hydrogen peroxide leads to anaerobic conditions and causes mass

mortality [10]. Low light intensity and high ocean temperature significantly reduce photosynthetic efficiency [11].

Reports from Intergovernmental Panel on Climate Change (IPCC) showed that emissions of toxic GHGs into the environment are rapidly increasing [12], even after the Paris Agreement, to reduce world temperature to 1.5 °C by 2100 and secure life on Earth [13]. Scientists have predicted that the warming effect will continue for about half a century [14]. This climate change poses serious threats to wildlife biodiversity as well as other living things, including humans [12, 15]. About 1/3 of human CO₂ emissions enter the oceans, leading to higher acidity (26 %) of the upper layer. This increase was expected to be 100 % or more by 2100 [12].

Abiotic stress has severe effects on all plant and animal species [16-19]. The biodiversity of various marine ecosystems has been disturbed due to global temperature rise [20]. Nitrogen is one of the main pollutants that directly or indirectly increases the temperature of marine and terrestrial ecosystems, thus affecting biodiversity. N₂O increases eutrophication and thus disturbs aquatic biodiversity [21]. The variability of coastal marine species is affected by the increase in ocean temperature [22]. The accumulation of algal toxins leads to the death of many aquatic species [23]. The toxic effects of various direct and indirect pollutants on the marine ecosystem are shown in Figure 1. To date, there is a lack of comprehensive literature on the effects of climate change on the marine ecosystem. Therefore, in this review, we focus on the negative impacts of climate change on marine biodiversity. Here we discuss the impacts of

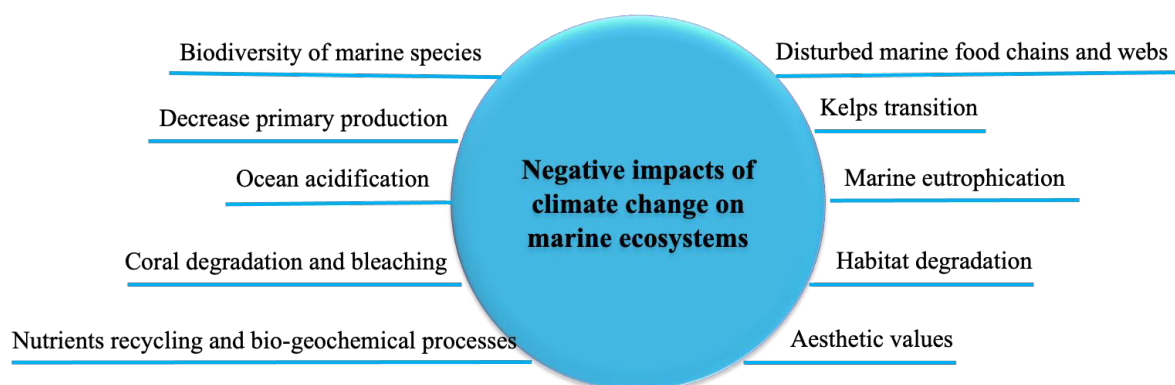


Fig. 1. Negative impacts of direct and indirect pollutants on marine ecosystems

climate change on plants, animals, and other marine species.

2. NEGATIVE IMPACTS OF CLIMATE CHANGE ON SPECIES DISTRIBUTION AND DIVERSITY

The change in climatic conditions may lead to the migration of wildlife [5]. Temperature growth and high salinity in the polar ocean disrupt the distribution of important taxa and ecosystems [24, 25]. High water temperatures can cause genetic divergence in both tropical and subtropical areas [26]. The frequency of small plants is crucially affected by these environmental changes [27]. Under these conditions, the marine reservoir system can protect the static organism [28]. Such a system is also useful in promoting population connectivity [29]. The reservoir system enriches genetic variability by increasing the size of the population and also provides an optimal environment [30]. In 2003, a high rate of the shoot mortality rate of important *Posidonia oceanica* was recorded by Marba and Duarte [31].

In accordance with previous reports, the increase in acidity in water bodies poses a serious concern to marine organisms [32, 33]. Declines in populations of plankton and other marine species, such as mollusks and calcareous algae, among others have been noted [34]. Some marine plants encompassing mangroves and seagrasses, help to minimize CO₂ emissions and increase pH; thus, these act as a defense system for some important marine calcifying organisms [35, 36]. These species are highly threatened and rapidly becoming extinct [37]. Therefore, the protection of this wetland from harmful human activities is necessary [38]. The conservation of the marine ecosystem helps to increase the population of teleost fishes. This type of fish plays a key role in the inorganic carbon cycle [39]. According to Graham and Harrod [40], ocean warming affects the genetic variability and function of many important ecosystems. It affects all levels of life, i.e., individuals, population, and community.

The rise in sea temperature also increases the severity of the disease. The effects of *Vibrio shiloi* on the coral *Oculina patagonica* increase with the rise in temperature [41]. Sometimes, ocean warming contributes to the release of some toxic

chemicals and causes serious health problems [42]. High temperatures also disrupt the chemical defense system of many marine animals [43]. Climate change in the ocean can also disrupt the biodiversity of local species [42]. Such warming leads to the extinction of cold-water boreal species [44]. Lower pH and highly dissolved CO₂ affect the biodiversity of the Mediterranean Sea. Acidification affects coralline red algae that serve as habitat and coral reef formation [45]. The immigration or transfer of invasive alien species disrupts existing species in many ways, such as displacing native species, losing genetic diversity, and affecting both habitat and community structure [46, 47]. Many invasive bivalves have negative impacts on native species [48]. The jellyfish *Rhopilema nomadica* has multiple negative impacts on marine ecosystems [49]. The Atlantic Percnon *gibbesi* has spread into the Mediterranean Sea and uses algae in large quantities for its energy production. It also competes with other native species for food and shelter [47].

Arctic species are so sensitive to abiotic factors that their abundance decreases significantly during Arctic summers [50, 51]. However, these responses vary with different factors such as nutrient availability, temperature increase, and ambient inconsistency [52]. Shellfish and the breeding process of other fishes are highly affected by high-temperature stress [53], the drop-in ocean pH [54], and the process of deoxygenation [55]. Pollution of the ocean from land and sea level rise is affecting the performance of reefs [56].

3. EFFECT ON PRODUCTIVITY AND OXYGEN DEMAND

The temperature rise can disorder normal metabolic and photosynthetic activities [57]. It also unsettles prey-predator ratios and reduces the overall mass of plant and animal species. The warming of the upper layer degrades the ability to mix nutrients and produce important products. The high stratification rate reduces oxygen levels and creates anaerobic conditions that lead to high mortality rates [57]. Fishing mortality rates have been increased by the unavailability of optimum oxygen demand [58] or by decreasing the total mass of phytoplankton and the low availability of essential nutrients [59]. General circulation models have indicated that dropping oxygen levels, along with other factors

such as eutrophication and overfishing, are a problem for marine ecosystems. A high mortality rate has been found in the deep lower parts of the oceans off the west coasts of North America [60] and southern Africa [61].

The lower oxygen concentration in the ocean disturbs multicellular organisms [62]. This concentration has decreased since 1950 due to the increase in ocean temperature [63]. The increase in atmospheric CO₂ concentration decreases the amount of O₂ [64]. Mobile species can survive at low oxygen levels, but inactive organisms are sensitive to low O₂ or die [65].

4. IMPACT ON CORAL AND CORAL REEFS

Coral reefs have been in severe decline due to human activities. In the last 200 years, the destruction of coral reefs has multiplied due to overfishing and water pollution [66-68]. Warm-water reefs have decayed by about 50 % in tropical locations over the past 30-50 years [69-71]. Cold-water reefs have also declined at a similar rate due to many factors such as cable and pipeline connections, trash accumulation, sampling, reef exploitation, and more [72, 73]. The biggest problem is the development and advancement of technologies used to exploit marine ecosystems [74].

The cold habitats for plants or animal species and coral reefs are endangered and there is a possibility that these may become extinct in the future due to ocean warming [57]. Coral reefs are very vulnerable to ocean warming and acidification [75]. In the last three decades, many coral reefs have been lost because of ocean pollution, overfishing, and temperature rise. It is noteworthy that if this temperature rise is not controlled, it may lead to a significant loss in coral reef production by 2050 [50, 76]. Therefore, temperature stress inhibits coral growth and calcification capacity [77] and promotes degradation and susceptibility to storms [78]. The decline in coral reef formation has a direct impact on fish production, which thus leads to poverty and food insecurity. This alteration may also decrease the population of some important fish species [79, 80]. Continuous and sudden changes are occurring in ocean ecosystems, which are interlinked with the disruption of the normal life of many vital marine species. Coral reefs are major sources of food,

shelter, and habitat for multiple marine organisms. In the deep sea, these reefs are the main habitats for numerous fishes. It must be highlighted that the rapid extinction of these coral reefs can cause serious problems for about 500 million people who rely directly on them for food, income, shelter, and other purposes. In some cases, this can cause regional insecurity [81].

The response of corals varies according to the type of environmental stress. The mutualistic relationship between warm water reefs is influenced by the physical/chemical changes in the environment. Thermal fluctuation or the presence of toxic compounds such as cyanide can distress this synergistic relationship [81, 82]. Similarly, coral bleaching has a damaging propensity to the progressive interaction between corals (Scleractinian) and Symbiodinium. Temperature surges over prolonged periods can promote mortality and bleaching [82, 83]. Bleaching of corals lessens their energy requirements and obstructs normal physiological function [84]. Previous studies have shown that changing climatic conditions can directly influence coral mortality and bleaching [85]. However, a high mortality rate was found in the early 1980s due to ocean warming caused by human activities [68]. The lack of symbiotic relationships among coral tissues caused serious threats due to starvation, failure of reproductive output and decrease in photosynthetic efficiency, poor competition, and diseases [68, 82, 86].

5. IMPACT ON THE MICROBIOME AND OTHER INTERACTION PROCESSES

Climate change is causing an increase in temperature and acidification, which affect the physiology and interactions of marine species. These climate changes have serious implications for kelp and its relevant microbiome [87]. The change in the structure and function of the microbiome is important in terms of environmental change. Seagrass species divide into similar rhizospheric communities [88]. Root structure and function have shown lowered exudates and thus lowered microbial populations in *Halophila ovalis*, *Halodule uninervis*, and *Cymodocea serrulate* [89].

One of the recent shotgun genomic studies has revealed that this process fosters the mutualistic

interaction of *Saccharina japonica* with the associated microbiome [90]. Some bacteria release toxic compounds that reduce the growth of other microorganisms on the surface of the alga [91]. At low salinity, some bacteria attach to species (*Ectocarpus*) and enhance their adaptive capacity [92]. The change in climatic conditions affects 1729 fish, 124 marine mammals, and 330 seabird species from 6 marine regions. Unfortunately, the world's very rich marine biodiversity is being seriously affected by ocean warming [74].

6. IMPACT ON MARINE SPECIES DISTRIBUTION AND BIODIVERSITY

Human activities are disrupting chemical processes, plant and animal species, and ocean temperatures [93]. Global warming affects the marine ecosystem in two ways. First, sea level rise reduces the area of wetlands [94]. These effects are particularly pronounced for mangroves [95]. Second, high temperature causes many important plant and animal species to shift to the pole by disrupting their habitat [96].

Regime shift has been observed in marine systems [93]. Regime shifts have been investigated in many studies, but these studies are limited to specific areas such as Florida Bay and the Baltic Sea [9]. In one of the condensed studies by Rocha *et al.* [97], 15 regime shifts were recorded and an efficient database was developed. These researchers have used the term “drivers” for all-natural or human-induced factors that affect our marine ecosystem through direct or indirect sources. The direct source affects the marine ecosystem processes while the indirect source affects the direct drivers [98]. Regime change is due to a variety of factors, with an average of 12 factors. One of the most important factors is the decline of mangroves [99]. The second most important factor is the eutrophication of the marine ecosystem [100], followed by changes in corals [101].

Temperature increases of up to 4 °C is disrupting populations and other important features of marine ecosystems [102]. Warming of the marine system is affecting algal and seagrass species, which are a source of protein and food for many marine species. A large number of these species are being lost or relocated to polar regions. Genetic adaptability

is slow due to increasing human pollution. Both epigenetic and non-genetic processes are required for proper adaptation and have direct effects on the phenotype, stability, and fitness of a species. Morphological variations are related to several physiological traits, reproductive success, and relationships with other organisms. The full study is based on the morphophysiological responses of algae and seagrass, which are important for appropriate conservation under climate change conditions [103]. High temperatures cause the extinction of many herbivore species and also disrupt the algal population. Moreover, it affects the success rate of many marine species [104].

An increased temperature causes the photosystem II (PS II) response and inhibits photosynthesis [105]. Increased ocean temperatures decrease total chlorophyll content and lead to coral bleaching along with the browning of seagrasses [106]. Similarly, ocean warming decreases both chlorophyll and carotenoid content of dwarf eelgrass (*Zostera noltii*). Temperature stress causes chlorophyll degradation and decreases the ratio of chlorophyll “a” and “b” [103, 107]. It also affects gametogenesis and the fertilization process of *Laminaria digitata* [89]. Ocean warming also causes the death of *P. oceanica* seeds [108]. High ocean temperature disrupts seedling performance. Moreover, it retards growth and increases leaf necrosis, mortality, and respiration efficiency of *P. oceanica* seedlings in the northwestern Mediterranean [109]. Temperature above 29 °C has caused the death of *Z. japonica* seedlings [110].

Frainer *et al.* [111] characterized the effects of climate change on 52 Arctic fish species of the Barents Sea for 13 key functional traits. They found that many functional traits were being replaced by some boreal species traits that were large, have longer lifespans, and were often piscivorous. Overfishing and maximum temperatures are two major extremes affecting many marine species. The poleward movement of marine species in response to warming is higher than in terrestrial ecosystems due to the reduced availability of physical shelter [80]. In addition, the lack of suitable habitat and other antagonistic interactions promote temperature-driven poleward movement [112]. Under these conditions, the response to this movement varies among genotypes. Some genotypes are strongly

affected by this change compared to other species [113]. This type of movement is more pronounced in mid to high-latitude warm-water species and therefore disrupts their important function and genetic variability [114].

However, the genotype response varied depending on the type of stress. Some genotypes showed themselves immune to global warming, while others remained sensitive and exhibited a low survival rate. For example, some fish species change their movements and move in both horizontal and vertical directions and have the ability to reproduce. Others, however, do not change direction and these species are expected to become extinct soon [115]. High CO₂ decreased the feeding ability and survival rate of crabs at a pH drop (0.3 units). At high CO₂, muscle consumption decreases by 50 %, and mortality increases (>50 %). The whelks showed resistance to elevated temperature and CO₂ stress. Abalone mussel growth decreased with increasing CO₂ levels. In the presence of crabs, the feeding of abalone was decreased and increased in warm water. Abalone mussel growth was significantly reduced by high CO₂ and low pH [116].

7. EFFECTS ON OTHER ECOSYSTEM FUNCTIONS

Temperature has a direct effect on the kinetic energy of molecular processes, such as the transport of materials to the membrane and enzymatic activities [117]. A slight change in temperature affects metabolic processes that directly impact various ecosystem functions and population growth [118]. Some organisms can acclimatize to these environmental changes within a limited temperature range. Once the limits are exceeded, the organism can no longer adapt, resulting in increased mortality, decreased fitness, and depleted population growth. Animal metabolism is also dependent on optimal temperature [117], in particular, prey-predator relations are affected by the increment in temperature [119]. Respiration rate reduces with increasing temperature; therefore, organisms require more calories to survive [5, 120]. Mesocosm investigations have shown that temperature elevation from 21 °C to 27 °C cutbacks biomass of the food web and plants and animals [121]. The size of phytoplankton also decreases due to warming [5].

8. OTHER EFFECTS

Climate change has positive, additive, or detrimental effects on certain marine species [34]. It becomes more dangerous by other factors such as overfishing, increases in nutrient levels in waters, and habitat alteration [122, 123]. However, the large population allows for reducing the likelihood of extinction at specific and general levels [124]. Under these conditions, marine reserves help to maintain the diversity of life and reduce the mortality rate of highly varied populations [125], thus enhancing the recovery process. Such reserves minimize the risk of outbreak and reduce prey growth [125, 126]. Variability among living organisms and the profusion of life has a direct impact on the ecosystem [127]. When maximum diversity is present in a reef ecosystem, it triggers more mass assemblages. In addition, more diverse communities may be less disturbed by maximum temperature stress [128]. The skeleton of corals releases calcium carbonate, which serves as a habitat for fish and other marine animals. These carbonated structures prevent biological and physical erosion. These reef ecosystems are believed to be the natural habitat for about 25 % of fish species [129].

The change in climatic conditions is troublesome to the normal processes of many important marine organisms by negatively affecting the habitats of marine species such as corals, seagrasses, oysters, and many others. Due to high temperatures, the mortality rate of corals is continuously increasing, by decreasing the presence and density of coral reefs and other species [130]. Deforestation of mangroves is growing rapidly and a loss of 10-20 % of mangroves was expected by 2100. This has a drastic implication for other marine ecosystems [131]. Sea ice or coral reefs play a prominent role in the genetic diversity of the marine ecosystem. It is fundamental for various activities of birds and animals and provides habitats for reproduction, migration, and other functions [132]. Due to climatic change, the penguin population is declining rapidly [133].

Climate change plays a central role also in affecting biological events [134]. Of note, these inconsistencies have been determined for gene expression [135], and in allele frequencies of the short-lived species [136]. Musculature

development is also linked to temperature [137] which has a straightforward effect on the movement of organisms, disrupting their speed and potential to compete with prey [138]. In a broader perspective, warming can damage physical behavior and increase the mortality rates of species [139]. It also disturbs community size and biomass [140]. Ocean warming distracts the growth rates of many marine species [141]. The formation of juveniles and larvae stages is linked with temperature rise [142]. Juvenile marine species are highly sensitive and cannot tolerate extreme temperatures [143]. Climate change in the oceans has direct effects on host vulnerability, the production of various pathogens, and the production of more disease vectors. An example of some of the diseases includes extreme temperatures causing diseases described in reef-building corals [144]. Another example is the increase in the severity of the deadly infectious disease (red abalone) in California [145]. One of the serious threats to the ocean ecosystem is the changes in geochemical processes due to the increase in acid concentration. This type of change leads to the loss of habitat, species distribution, community composition, and other interactions described elsewhere [146].

9. IMPACTS OF CLIMATE CHANGE ON GLOBALLY IMPORTANT MARINE ECOSYSTEMS

High-temperature stress has significantly impacted both plant and animal species of the Arabian Gulf [147, 148]. Continued climate change is disrupting the Gulf's marine biodiversity. The high salinity and temperature stress determine the tolerance of species [149]. The occurrence and diversity of species in the Gulf marine ecosystem are low [150, 151], but from a biological perspective, these species have many values [152]. The environmental extremes such as the increase in world temperature and sea level, decrease in oxygen levels and other human activities such as overfishing, oil exploration, etc. are disturbing the biodiversity of this region [153, 154].

Marine species of the Gulf are more tolerant to heat stress compared to other parts of the world [155, 156]. The temperature increase has been determined to be up to +0.57 °C in the Gulf between 1950 and 2010 [157] has affected many marine

species [158]. The temperature increase (35-37 °C) has more than quintupled the frequency of coral bleaching since late 1990 [159]. These impacts are very severe and in recent decades about 70% of the reefs in this region have disappeared within a few decades. Such mass mortality has a direct impact on other fish species [160]. Loss of hawksbill sea turtle habitat is expected in various parts of the Gulf. The rate of extinction is highest in the southwestern part of the Gulf, off the coast of Saudi Arabia, Qatar, and the UAE. The fisheries of Iran and Qatar may be more susceptible to climate variability [161].

Mediterranean Sea has a rich marine diversity that is affected by climate change [162]. The Mediterranean ecosystem has been reported with greater effects by habitat alteration, environmental change, and pollution, overexploitation of key species, introduction of unknown species into pre-existing biota, and by coastal urbanization [42, 163]. Environmental changes affect marine organisms in a variety of ways. Species with low biodiversity are more exposed to these threats and may become locally extinct [42, 164]. Heat and drought stress also this region [164, 165]. In 1999 and 2003, climate change led to the mass mortality of many species of benthic invertebrates [166].

Climate change has serious impacts on all small island states through temperature and sea level rise and changes in rainfall frequency. The effects of sea level rise are extremely dangerous for coastal ecosystems. The presence of large numbers of people on the coast disrupts normal sea levels, flooding, erosion, and the availability of trash disrupts small Caribbean Islands. These changes directly affect the habitats of many marine species and also lead to ecosystem degradation. Such environmental changes in the Caribbean Island increase disease and the production of various parasites, as well as the immigration of new species into the native biota [57, 162]. Many fish species are seriously threatened in Caribbean marine systems. The increasing biomass of algae also affects different ecosystems. Algal blooms reduce the availability of nutrients and increase ocean temperatures [162]. In addition, the opossum shrimp is greatly affected by the increase in temperature by causing anaerobic conditions in the Irish glacial relic *Mysis salemaai*. High temperature degrades habitat quality and slows down the survival rate of mammal species

[115]. The high sea temperature increases the alien invertebrates and fish species of the Israeli Mediterranean Sea. In contrast, populations of native fish and endangered species are declining [167].

10. RISK MANAGEMENT IN A CHANGING MARINE ECOSYSTEM

Greenhouse gas concentrations in the atmosphere can be controlled in many ways, including the use of renewable energy, increasing energy production capacity, carbon sequestration and safe storage, and enhancing natural carbon resources [15]. Most policies are based on the country's response to climate change [165], while ocean-based policies receive little attention. Therefore, ocean-based policies are mandatory to prevent ocean warming, control acidification, lower sea levels, and protect habitat degradation.

Climate changes induced by the ocean system are a major concern for policy makers and managers. The lack of effective planning and policy reduces the available resources for different marine species. "No regret" policies are necessary to keep the oceans safe for all marine species. Deforestation of important plant species like mangroves should be controlled to maintain the optimum temperature of the oceans. There is a need to manage or efficiently use available natural resources and prevent the release of toxic greenhouse gases into the upper or lower layers of the ocean [5, 168] described that humans can disrupt the marine ecosystem in five ways, namely by increasing acidity, raising sea level, increasing storms, disrupting the occurrence and diversity of species, and lowering oxygen levels. They emphasized that not only the release of CO₂ should be minimized as per the 2015 Paris Agreement greenhouse gas emissions, but also some other management and implementation steps are needed to protect marine flora and fauna.

With the increasing pressure on the marine ecosystem, it is now important to develop effective planning and policies to improve and safeguard ocean/deep-sea biodiversity. But still, there is a lack of proper strategies and good governance to tackle such issues and enhance the life system of many important marine organisms. In many parts of the world, governments have not given full authority

to environmental protection agencies, which is one of the major problems for marine ecosystem security [169]. Preference is given to anthropogenic activities at certain experimental sites, e.g., fishing/mining. However, natural or other human activities that affect the ocean ecosystem are not preferred [170, 171]. Clear evidence of the lack of adequate management is the fact that 64 % of the ocean area is beyond national jurisdiction (ABNJ) [171]. In particular, there is no single authority for the protection of the deep sea that is committed to its safety and biosecurity [172].

An important step towards safeguarding marine biodiversity is the establishment of Marine Protected Areas (MPAs). MPAs will protect many important and diverse marine organisms by protecting their habitats and increasing their abundance. Thus, MPAs will contribute to the conservation of many important marine organisms. This system requires effective planning, management, and implementation strategies [173, 174]. Specific conservation strategies, including fully and strongly protected areas, help and maintain marine biodiversity. According to the Convention on Biological Diversity and Sustainable Development Goal 14, coastal states are far beyond their target, having committed to protecting 10 % of the waters [175]. Some recent studies suggest that the target for MPAs should be 30 % to optimally protect marine ecosystems [176]. The MPA system needs to identify the gaps and should close them within deadlines. This process can be further improved through dialogs and the development of a clear agenda to safeguard the lives of marine species. But this process is not very successful due to the lack of human resources, funding sources, availability of equipment [177], and lack of discussion and help from local people. After a healthy dialog between marine researchers and managers, they concluded that not only MPAs but also efficient fisheries management strategies are important for about 70-90 % of the oceans [178].

CO₂ concentrations in the air increased to 406 ppm by November 2017 [179]. The Paris Climate Agreement of 2015 [180] set the main goal to limit the global temperature increase to below 1.5 °C. To achieve this goal, regular and efficient methods of removing atmospheric C are required [181]. According to Johnson *et al.* [182], the North

Atlantic deep-water area and open oceans will be severely affected in the next 20-50 years. More and more efficient oceanographic data are important to review and calculate the species. In addition, more research is needed to review the changes in the deep-sea ecosystem and response times. They recommended precautionary measures and minimization of harmful human activities to the ecosystem that directly affect the deep-sea system. Vulnerability and participatory assessments are important to minimize the risk of extinction of many important marine species [161]. Abiotic techniques must be used to remove CO₂ from seawater [183, 184]. Marine spatial planning techniques must be used to conserve or save a large proportion of marine species [185]. Regime change requires international rules and regulations to minimize the risk to the marine ecosystem [97]. The possible solution to protect the marine ecosystem from direct and indirect pollution is shown in Figure 2 modified by Gattuso *et al.* [33].

Epigenetically driven variability plays a vital role in optimizing species in the face of changing environmental conditions [186, 187]. DNA polymorphism helps in slow acclimation processes under changing climatic conditions [188]. Epigenetic mechanisms increase speciation, acclimation, and plasticity under extreme environmental conditions [189]. In addition, the methylation process aids in species adaptation [190]. The network enhances DNA methylation by regulating metabolic pathways at elevated temperatures (-1.5 °C vs. + 4 °C) [191]. Similarly, DNA methylation occurs at the larval stage when temperature increases up to 2 °C in the European seabass [192]. Acidification aids in the morphologically based adaptation of the

coral *Stylophora pistillata* [193]. DNA methylation occurs in red, green, and brown algae, as well as diatoms [194, 195]. In addition, CpG islands and jumping genes enhance adaptation traits [196]. In seagrass (*Zostera marina*), jumping gene disruption occurs, forming a new promoter and splice sites, reactivating gene function, and enhancing acclimation to high temperatures [197]. Clonal growth together with a mutation in somatic cells plays a role in the adaptation and fitness of marine species to climate change [198].

11. CONCLUDING REMARKS AND FUTURE PERSPECTIVE

High CO₂ and ocean warming have serious implications for the biota of marine ecosystems, including important plant and animal species. Ocean warming disrupts the morpho-biochemical and physiological processes of marine species. The effects on corals, which serve as shelter, habitat, and nutrient sources for many important marine species, are particularly severe. If these changes continue at the same rate, a large number of marine species will become extinct in the near future. Therefore, it is necessary to protect wetlands from anthropogenic activities, avoid toxic greenhouse gasses, and promote the efficiency of CO₂ precipitation in terrestrial ecosystems. In addition, serious steps must be taken to minimize man-made pollution. Thus, it is the responsibility of every individual, institution, government, and non-governmental organization to develop effective planning and policies for the safe protection of marine species. Strict codes and “No regret” policies should be implemented all over the world to protect the biodiversity of the various marine ecosystems.

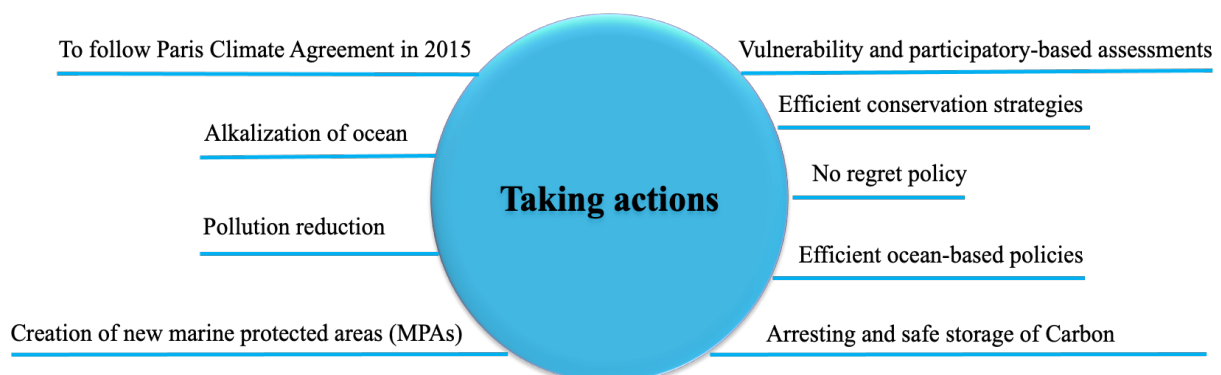


Fig. 2. Salient actions to protect marine ecosystems

12. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Production of Fibrinolytic Enzyme by Soil *Actinobacteria*

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Abstract: Thrombotic diseases are increasingly becoming among the prominent causes of death across the globe. Researchers are now turning attention towards fibrinolytic enzymes as potential alternative treatment for thrombotic diseases. This present study focused on the production of extracellular fibrinolytic enzyme from soil *Actinobacteria* and evaluation of its hydrolytic activity on blood clot. The *Actinobacteria* was isolated from compost soil of semi-arid climate in Nigeria. Bacterial isolation was achieved using starch casein agar. Potent fibrinolytic enzyme producing *Actinobacteria* were identified and subjected to enzyme production using submerged fermentation method. The interactive effects of incubation time, temperature, pH and media components on enzyme production were analysed. Extracellular fibrinolytic enzyme produced by the selected *Actinobacteria* was partially purified by ammonium sulphate precipitation and subsequently assayed for blood clot lysis activity. Results of these studies indicated that fibrinolytic enzyme was produced optimally at pH 8 and temperature of 40 °C after 72 hour of fermentation. Partially purified fibrinolytic enzyme was able to degrade blood clot comparable to the positive control. These results shows that soil *Actinobacteria* of unexplored semi-arid climate of Nigeria present a prospect in search of novel microorganisms with potentials in the production of fibrinolytic enzyme that can serve as an alternative blood clot buster in treating thrombotic diseases.

Keywords: *Actinobacteria*, Semi-Arid, Fibrinolytic Enzyme, Yield optimization, Thrombolytic.

1. INTRODUCTION

Myocardial localized necrosis occurrence is on the increase globally, primarily due to Thrombosis and pulmonary embolism that develop inside the arteries [1, 2]. Thrombosis is a critical event and a common pathology underlying myocardial infarction [3]. Treatment of thrombosis is the usual way through which acute myocardial infarction (AMI) is combated. The use of synthetic therapeutics in treating thrombosis can result to negative effects such as allergic response, short half-life and they are expensive to acquire [4-6]. Therefore, it is critical to research and develop natural, safer, and cost effective thrombolytic agents. Nowadays, protein treatments are commonly used in combating AMI. In the last decade, microbial fibrinolytic proteases

have shown great potential for therapeutic application [7]. It is envisaged that fibrinolytic enzymes of natural source are less likely to induce negative effects such as allergic response and they are inexpensive to produce. Therefore, therapeutic screening of fibrinolytic enzymes from microbial origin could be the most suitable method for fibrinolytic enzymes production [7].

Actinobacteria are widely distributed in nature inhabiting mainly soil and plants. They are filamentous Gram-positive microorganisms that are ubiquitously distributed in nature. They are significantly an important sources of bioactive metabolites of industrial and biotechnological importance [8]. There are literature reports on the production of fibrinolytic proteases by

the *Actinobacteria* from aquatic and terrestrial ecosystems [9-13].

The expanding applications of fibrinolytic proteases motivated this study to screen for novel fibrinolytic *Actinobacteria* from soil. One of the advantages of producing enzymes from microbial sources is that, it could be achieved using low cost substrates. From the industrial point of view, designing of a low cost production medium for bio products such as enzymes is one of the curial factors, because the market price of the particular bio products is directly influenced by the cost of production medium components. Therefore, utilizing a locally abundant cost effective inexpensive substrates could reduce the cost of production [14]. The present study aims to isolate soil *Actinobacteria* and induce extracellular fibrinolytic enzyme production by the isolated *Actinobacteria*. To the best of our knowledge, this is the first attempt to explore the potential of soil *Actinobacteria* of slaughter house for fibrinolytic enzyme production and characterization.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil sample was collected on 25th March 2021, from the abattoir compost soil in Yeldu town (12°47'06''N 4°15'56''E) at the depth of approximately 12 cm from the surface and brought to the laboratory in sterile polythene bag. Soil samples were incubated at 80 °C for 15 minutes to eliminate most of the unwanted gram-negative bacteria. Soils were allowed to cool and then 1 g of each soil sample was treated in a two-step process with 0.1 g CaCO₃ followed by 1.5 % phenol for 30 min.

2.2 Isolation and Identification of *Actinobacteria*

About 1g of the treated soil samples were suspended in 9 ml normal saline and vortexed. Serial dilution was carried out up to 10⁻⁵. Then 100µl of the serially diluted sample from 10⁻⁵ tube was inoculated on starch casein agar (SCA) supplemented with 50 µg/mL cycloheximide and 50 µg/mL nalidixic acid to restrict fungal and gram negative bacteria growth respectively. The resultant colonies were identified as *Actinobacteria* using conventional microbiology techniques and subsequently sub-cultured to obtain pure cultures. Screening for

casein and fibrin hydrolysis was carried out based on the maximum zone of hydrolysis in both casein and fibrin, and the potent isolate was selected for further investigation [15-17].

2.3 Fibrinolytic Enzyme Production and Optimization of Production Media

Fibrinolytic enzyme production was initiated by transferring a single colony of *Actinobacteria* from the casein agar plate into a Erlenmeyer flask containing 50 mL of casein basal broth (Casein 10 gL⁻¹; K₂HPO₄ 2 gL⁻¹; KNO₃ 2 gL⁻¹; NaCl 2 gL⁻¹; Casein 0.3 gL⁻¹, and 0.02 gL⁻¹ of MgSO₄, CaCO₃, and FeSO₄ each, pH 7.5) [18], and incubated for 18 hrs at constant shaking of 200 rpm. The culture was then diluted at 1:100 (v/v) with the same but fresh Casein broth for three successive passages. Then 5 ml of the passaged culture of *Actinobacteria* was inoculated into another 250 ml of Casein basal broth, then incubated at 37 °C for 5 days on a shaker at 200 rpm.

To determine the required optimum physicochemical conditions in achieving maximum yield of keratinase through submerged fermentation (SF), a one-variable-at-a-time (OVAT) method was adopted and the effects of incubation period (24–120 h), incubation temperature (25–60 °C at 5 °C interval), pH (5.0–9.0), carbon and nitrogen sources were evaluated for optimal production of keratinase. Each experiment was carried out in triplicates. After 5 days of incubation, the crude extracellular fibrinolytic enzyme was obtained as a supernatant by centrifugation at 7,000 rpm for 10 min at 4 °C and then subjected to 85 % saturation of ammonium sulphate (NH₄)₂SO₄. Using a dialysis tubing cellulose membrane obtained from Sigma-Aldrich, the ammonium sulphate precipitate was dialyzed against 10 mM Tris–HCl buffer (pH 7.5) [19].

2.4 Fibrinolytic Enzyme Assay

The reaction was setup with 3 mL of 0.1 M Tris- HCl containing 0.01 M CaCl₂ (pH 7.8), 1 mL (1 % w/v) of fibrin, and 1 mL of partially purified enzyme. The reaction was allowed to continue for 30 min at 37 °C after which 5 mL of trichloroacetic acid (TCA) containing 0.22 M sodium acetate and 0.33 M acetic acid was added to terminate the reaction. The

reaction mixture was centrifuged and absorbance of the supernatant was taken at 275 nm. One unit of fibrinolytic enzyme was defined as the amount of enzyme required to increase the absorbance at 275 nm equivalent to 1 µg of tyrosine per min [20]. The enzyme activity (EA) was calculated in U/mL according to the following Equation:

$$EA = (\Delta A \times V \times f) / (\epsilon \times l \times v_0) \quad (\text{Equation 1})$$

Where ΔA is the change in absorbance, V is the final volume of the reaction, f is the dilution factor, ϵ is the extinction coefficient, l is the path length of the cell and v_0 volume of the enzyme used.

2.5 *In vitro* Assay of Blood Clot Lysis

Goat blood was obtained from a slaughter house in Aliero town. The blood was allowed to form a clot and then washed with phosphate-buffered saline to remove any impurities before adding the fibrinolytic enzyme. Positive and negative controls were prepared with clexane injection and a 0.9 % w/v saline solution respectively. A 5 mL of fresh goat blood was used for each test and all reaction mixtures were allowed to stand for 3 h at room temperature and checking after every 30 min [20].

2.6 Statistical analysis

Triplicates tests were performed in all the experiments and results were presented as Mean \pm SD values. The significant differences of the data were evaluated via Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT). Findings of all the experimental tests were significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of *Actinobacteria*

Actinobacteria of the slaughter house compost soil were isolated and identified based on the colonies morphological appearance on starch casein agar plate. The colonies were mainly white and some tiny, spherical and irregular ash-grey (Figure 1A). The isolated *Actinobacteria* was able to hydrolyse casein protein in the medium, indicating its proteolytic activity. Distinct colonies were selected and sub-cultured (Figure 1B) to have pure colonies

which were subsequently subjected to production of fibrinolytic enzyme.

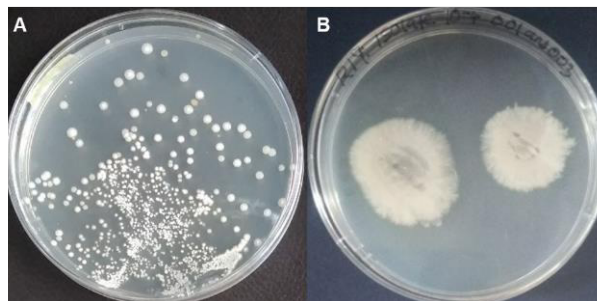


Fig. 1. (A) *Actinobacteria* isolates. (B) Sub-cultured *Actinobacteria* isolates.

3.2 Production of Fibrinolytic Enzyme and Optimization of Production Media

Various process parameters including incubation period, incubation temperature, pH, carbon and nitrogen sources influence fibrinolytic protease production. Depending on the microbial strains being studied, these production parameters varies for achieving maximum enzyme production. In this study, fibrinolytic enzyme production was initiated and allowed to continue for a period of 5 days under varying fermentation conditions including incubation period, temperature, pH, carbon and nitrogen sources. Experiments were carried out in triplicates under each fermentation condition. At 24 h intervals, an aliquot of the production media was collected and centrifuged. The supernatant was used as crude enzyme and the enzyme activity was assessed as described above.

Fibrinolytic enzyme production was determined by the activity of the crude enzyme, more activity indicate high concentration of the crude extracellular enzyme. Results of the OVAT experiments indicates that production of fibrinolytic enzyme started at 24 hour post inoculation and continued up to the fifth day, the enzyme production reached maximum (53.48 U/mL) at 72 h after which it begins to decline (Figure 2).

Previous study on *Alcaligenes aquatilis* PJS_1 reported 60 h incubation period as the optimum for maximum fibrinolytic enzyme production [14] whereas, *Bacillus* sp. IND7 was reported to achieve maximum production of fibrinolytic enzyme at 72 h [20].

Temperature is among the crucial factors that influences the ability of microorganisms to grow and synthesise metabolites. Each microorganism has its favourable temperature, therefore, metabolic reaction in microorganisms could be affected due to change in the fermentation temperature. This study assessed the temperature effects on fibrinolytic enzyme production by the isolated *Actinobacteria*. According to the results obtained, the optimum temperature for maximum enzyme production (68.28 U/mL) was 40 °C, and an increase in the incubation temperature to 45 °C causes a decline in the production of enzyme (Figure 3).

Previous studies on the production of fibrinolytic enzyme by marine *Actinobacteria* have reported the optimum temperatures for maximum enzyme production to be between 33 and 37 °C [12, 13]. Whereas, optimum fermentation temperature for fibrinolytic enzyme production by other bacterial strains was reported to be 35 °C [14, 21]. The variation in optimum fermentation temperature for fibrinolytic enzyme production between our study and the previous studies could be directly related to the fact that the sources of organisms and the bacterial strains are different from each study. In this study, the *Actinobacteria* was obtained from the compost soil of Yeldu town in Kebbi State, Nigeria which is a hot semi-arid climate where the daytime temperature can reach up to 45 °C during the warmest months of March to May. Hence, it is not surprising to have microorganisms from that habitat favouring high temperature for production of secondary metabolites.

Medium pH is another factor that affects the growth and synthesis of metabolites because metabolic reaction can be influenced as a result of change in the pH of the production media. The effect

of pH on the production of fibrinolytic enzyme by the isolated *Actinobacteria* was evaluated and the results indicated that maximum enzyme production (57.69 U/mL) was achieved when the pH of the production media was 8 (Figure 4). Previous studies on the production of fibrinolytic enzyme by marine *Streptomyces* reported that maximum enzyme production was achieved when the pH of the production media was between 7 and 7.3 [12,13]. This shows that *Actinobacteria* fibrinolytic enzyme production is more favoured in a media of between neutral to near alkaline pH.

Carbon and nitrogen plays a role in the synthesis of microbial metabolites, hence the need to assess how sources of carbon and nitrogen can affect production of extracellular fibrinolytic enzyme by the *Actinobacteria* isolate. Results of these evaluations suggests that the *Actinobacteria* isolate was able to grow in all the media containing different sources of carbon and nitrogen, although the level of enzyme production varies with different source. For the carbon sources, the highest enzyme production of 71.13 U/mL was observed from the fermentation media containing glucose as the only source (Figure 5). Whereas, highest production of enzyme 65.11 U/mL was observed in the presence of tyrosine as nitrogen source (Figure 6).

Previous study reported a maximum fibrinolytic enzyme production by *Streptomyces rubiginosus* VITPSS1 in the presence of glycerol and soybeans as carbon and nitrogen sources respectively [12]. In another study, *S. radiopugnans* VITSD8 was reported to exhibit the highest yield of fibrinolytic enzyme when peptone and maltose were the sources of nitrogen and carbon respectively. Other strains of bacteria were also reported to having high yield of fibrinolytic enzyme in the presence of different

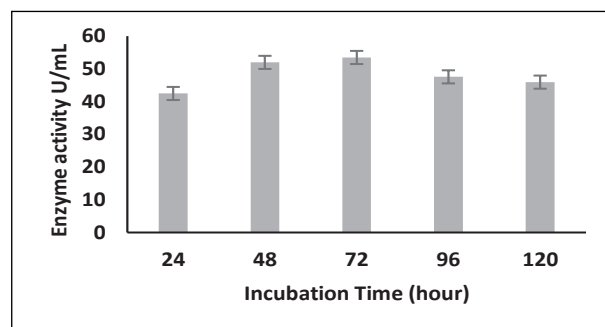


Fig. 2. Fibrinolytic enzyme activity of *Actinobacteria* isolate at different incubation period.

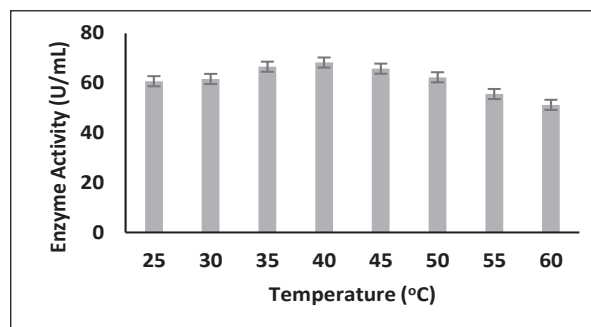


Fig. 3. Fibrinolytic enzyme activity of *Actinobacteria* isolate at different temperature.

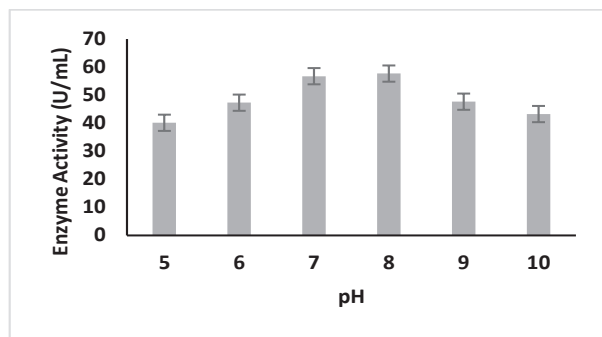


Fig. 4. Fibrinolytic enzyme activity of *Actinobacteria* isolate at different pH.

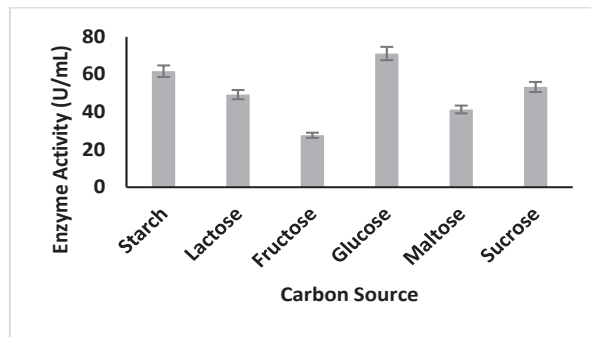


Fig. 5. Fibrinolytic enzyme production by soil *Actinobacteria* isolate under different carbon sources.

carbon and nitrogen sources [14, 21, 22]. This indicates that, the source of microorganisms and the strains can influence a variation in the synthesis of microbial metabolites as evidenced from these studies. This means that *Actinobacteria* of marine origin can be different from that of terrestrial origin in terms of factors that influence enzymes production, hence the need to explore more habitats in search of novel microbes for low cost production of fibrinolytic enzyme to convert thrombolysis disorder.

3.3 Partial Purification of Fibrinolytic Enzyme

The crude supernatant of the fermentation broth was partially purified with 85 % saturation of ammonium sulphate. From the purification results, it was observed that partially purified fibrinolytic enzyme exhibited a specific activity of 249.5 U/mg and 37 % yield after dialysis step (Table 1). The percentage yield was obtained using the formula

$$\% \text{ yield} = \frac{\text{Total activity after dialysis}}{\text{Starting total activity}} \times 100$$

(Equation 2)

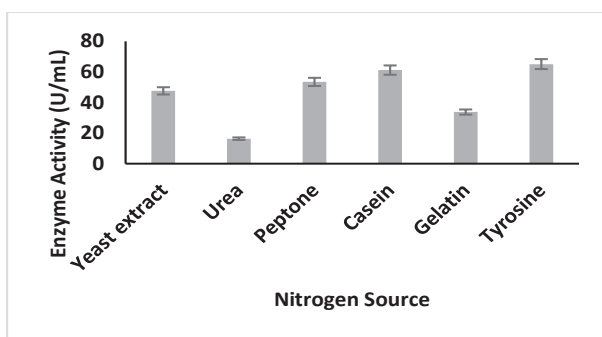


Fig. 6. Fibrinolytic enzyme production by soil *Actinobacteria* isolate under different carbon sources.

3.4 In vitro Assay of Blood Clot Lysis

Potential hydrolytic activity of the partially purified fibrinolytic enzyme (200 IU/mL) was evaluated on fresh goat blood in vitro. Clexane injection (200 IU/mL) and a 0.9 % w/v saline solution were considered as the positive and negative controls respectively. Results of these assays indicated that partially purified fibrinolytic enzyme achieved about 75 % clot lysis compared to 100 % achieved by the clexane injection (Figure 7).

Various studies have been reported on the blood clot lysis activity of fibrinolytic enzyme of microbial origins exhibiting different level of blood clot lysis. These include protease SFE1 [5], fibrinolytic protease of *Streptomyces rubiginosus* VITPSS1 [12], fibrinolytic protease of *Streptomyces radiopugnans* VITSD8 [13], the free protease BC1 and immobilized CLEA-Fib-mChi [23], fibrinolytic protease of *Serratia marcescens* subsp. *sakuensis* [24], and streptokinase [25]. Hence, our study further revealed the actinokinase-like activity of fibrinolytic enzyme from soil *Actinobacteria*. The fibrinolytic proteases are more

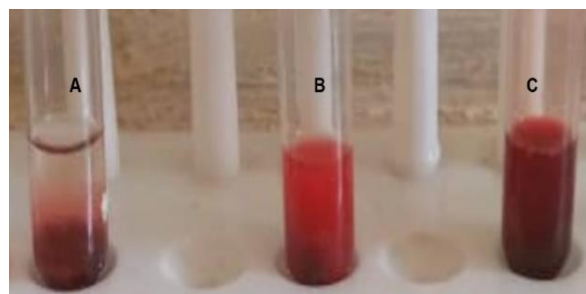


Fig. 7. Blood clot lysis activity. (A) Negative control (0.9 % saline solution) (B) Partially purified fibrinolytic enzyme (C) Positive control (Clexane injection)

Table 1. Purification table for fibrinolytic enzyme

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	% Yield	Purification Fold
Crude Supernatant	281	27,765.3	65.7	100	1
Ammonium Sulfate	85.6	19,429.2	151.2	46.6	2.3
Dialysis	41	10,229.5	249.5	37	3.8

of neutral enzymes that are produced mostly from *Actinobacteria*. Considering the high demand of fibrinolytic enzymes and the negative effects of the available drugs for the treatment of thrombosis, it is important to explore more fibrinolytic enzyme producing *Actinobacteria* especially from unexplored habitats. For the first time, the results of our study revealed that the soil compost of slaughter house from the semi-arid climate of Nigeria is a promising source of novel *Actinobacteria* with potentials in the production of fibrinolytic enzymes and possibly other industrially valuable secondary metabolites.

4. CONCLUSION

Actinobacteria was isolated from the compost soil of slaughter house obtained from Yeldu town and a proteolytic enzyme was produced by the isolated *Actinobacteria* strain. Due to its blood clot degradation activity in comparable with the standard clexane injection the protease was considered as fibrinolytic enzyme. It can be envisage that this fibrinolytic protease can be studied further towards developing it as a potential thrombolytic agent for treating heart diseases. Enzymes from unexplored natural habitats could be of immense benefits in various industrial applications. The findings of our study present more enlightenment that can lead to bioprospecting of fibrinolytic enzymes and even other enzymes of industrial importance from semi-arid habitats of Nigeria. We aim to further our study towards the molecular identification of the isolated *Actinobacteria* strain and carry out more purification steps to have enzyme of high purity in order to characterize the enzyme.

5. ACKNOWLEDGMENTS

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laboratories for the execution of this research work.

6. CONFLICT OF INTEREST

We declare no conflict of interest.

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Growth Dynamics and Resource Allocation of *Bistorta amplexicaulis* (D. Don) Greene: An Alteration across Different Habitats and Altitudes

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Abstract: *Bistorta amplexicaulis* is an essential medicinal plant found in the Kashmir Himalaya. Ethnobotanical studies have revealed that this particular species is used to treat fractures, muscle injuries, heart problems, abnormal leucorrhoea, menorrhagia and inflammation of the mouth and tongue. The current study aimed to determine the variation in growth traits and fluctuations in the allocation patterns with respect to different habitats across the altitudinal gradient. In order to adapt to unpredictable and stressful conditions at higher altitudes, phenotypic plasticity plays a crucial role. Our findings revealed considerable variability in the phenotypic traits, indicating that altitude has a defined effect on this species's morphology and reproductive traits. Low altitude plant populations of Kashmir University Botanical Garden (KUBG), Dara and Tangmarg were more robust and taller (98.4 ± 2.36 , 83.58 ± 2.69 and 74.08 ± 1.59 cm, respectively) than the populations of Pissu top and Bangus (23.96 ± 3.38 and 30.43 ± 1.12 cm respectively) at higher altitudes. The habitats of KUBG, Dara, and Tangmarg proved to be substantially better for the growth of *B. amplexicaulis*, as per the Principal component analysis (PCA). The regression analysis demonstrated a negative relation between altitude and plant height. Traits such as leaf length/ breadth, Rhizome length/ breadth and inflorescence length showed a strong correlation with plant height. Our results provide an inclusive description of the phenotypic variability of this significant medicinal plant in response to the habitat variability across different altitudes.

Keywords: Kashmir Himalaya, Phenotypic Variability, Habitat Variability, Altitudinal Gradient

1. INTRODUCTION

An alluring south Asian region- the Kashmir Himalaya is situated at the north-western tip of the Himalayan Biodiversity Hotspot. It harbors around 10,000 plant species, of which 4,000 are endemic, making up about 2.5 % of the global angiosperm diversity [1]. Due to their sedentary nature, plants cannot avoid the vulnerabilities of the environment in which they grow. Therefore, to persist in severe conditions, they must adapt genetically or by phenotypic plasticity [2]. As an adaptation to resource availability, plants can display disparity in morphological characteristics in response to abiotic (temperature, rainfall, and soil) and biotic (grazing

and competition) aspects of the environment. The competence of plants to transform their morphology and physiology as a response to inconsistent environmental conditions is called phenotypic plasticity; this phenotypic adaptation is also termed phenotypic accommodation [3]. Phenotypic plasticity plays a crucial role in evolution by adjusting the developmental pathways, thus leading to phenotypic diversity in nature [4]. Phenotypic plasticity has a crucial role in several aspects, such as resource acquisition by plants and differences in the size and positioning of resource-attaining parts (leaves), which are critical to a plant's regulation of resource accessibility.

Variability in general growth forms of resource accumulating organs such as tubers, roots, rhizomes, flowers and leaves of a plant are critical to regulate the available resources [5]. Phenotypic plasticity that is environmentally induced in plants is usually measured as a functional response that amplifies fitness in fluctuating environments. If environmental and phenotypic variation within species are correlated, it can be hypothesized that species are phenotypically more variable if they occupy an extensive range of habitats as supported by Sultan (2001) [6]. With increasing evidence from molecular and developmental biology, we have gained an advanced understanding of plasticity methods which are critical for tracking changes in specie distribution, the composition of a community, and the productivity of crops under climate change [7].

Bistorta amplexicaulis (D. Don) Greene (Syn: *Polygonum amplexicaule* D. Don), locally known as “machran chai” belongs to the family Polygonaceae and grows as a medium-sized herb in hilly areas of Kashmir [8]. It is a highly useful medicinal plant, native to China north-central, China south central, Afghanistan China southeast, East Himalaya, Pakistan, Nepal and West Himalaya (POWO). It is a highly useful medicinal plant, native to China north-central, China south central, Afghanistan China southeast, East Himalaya, Pakistan, Nepal and West Himalaya (POWO). *B. amplexicaulis*, a medicinally significant plant, has been used to cure many ailments, such as maintaining normal menstrual flow, reducing stomach pain, treating fractures, rheumatism, osteoporosis, muscle injuries and inflammation of the mouth and tongue [9, 10]. Phytochemical analysis of *B. amplexicaulis* has revealed numerous compounds, including, Friedelin, β -sitosterol, simiarenone, angelicin, psoralen, palmitic acid, quercetin [11] catechin, quercetin-3-O- β -D galactopyranoside, rutin, amplexicine [12]. The current research work aimed to reveal (i) Impact of altitude and habitat variability on the morphological attributes and allocation patterns of *B. amplexicaulis* (ii) to find a suitable habitat for the development, establishment and cultivation of *B. amplexicaulis*.

2. MATERIALS AND METHODS

2.1 Study Area

The Kashmir Himalaya constitutes a distinct

biosphere unit in the northern Himalayas due to its bio-geographically important location [13]. The region covers around 15,948 km² and is mountainous for nearly 64 % of its overall size. The region is located between 32°20' and 34°50' North latitude and 73°55' and 75°35' East longitude [14]. Geographically, the region consists primarily of an oblique bowl-shaped valley. The valley is encircled in the south and southwest by the Pir Panjal range of the Lesser Himalaya, and in the north and northeast by the Zaskar range of the Greater Himalaya.

2.2 Survey and Selection of Study Sites

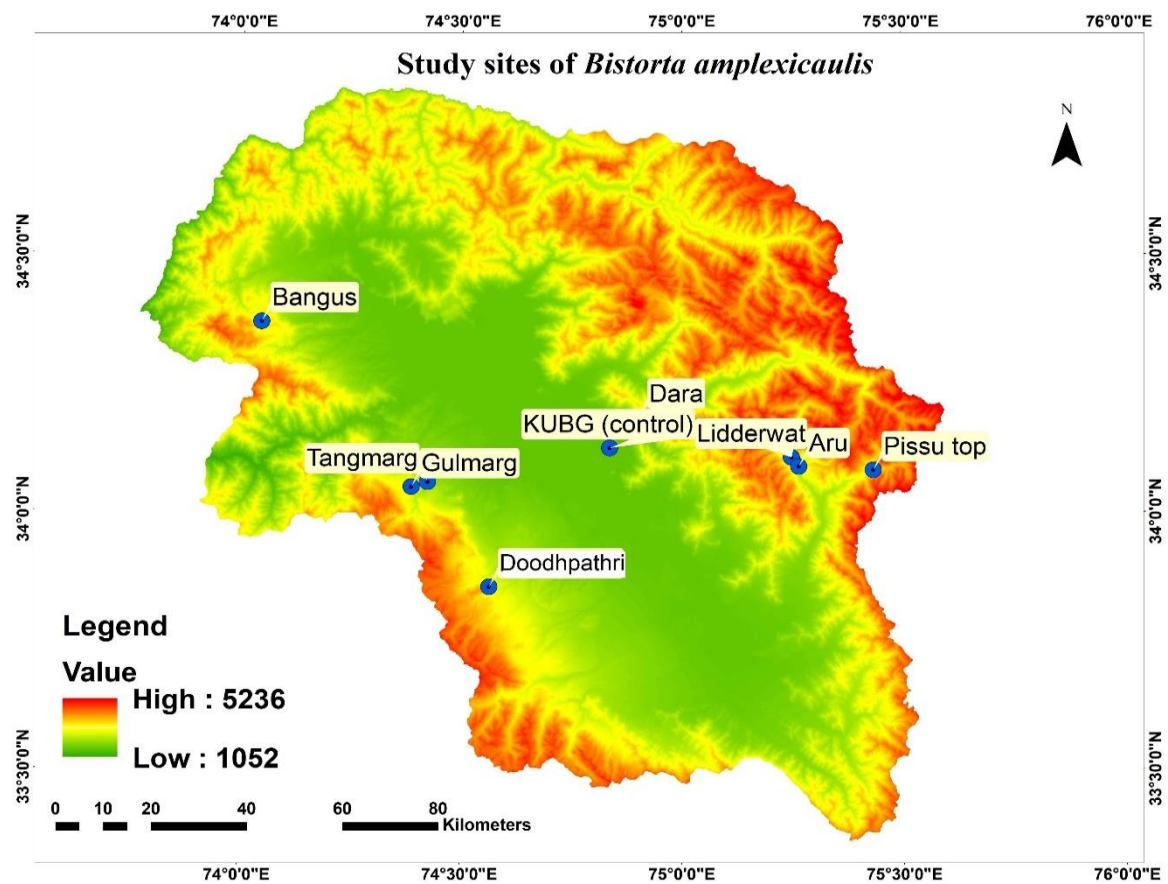
Extended field surveys were conducted during 2019-2021 in varied habitats of Kashmir Himalaya in order to recognize specific areas across diverse geographical conditions. *Bistorta amplexicaulis* was found growing in Dara, Gulmarg, Doodhpathri, Tangmarg, Sinthan top, Aru, Lidderwat, Pissu top, Tarsar, Tosa Maidan, Royelsar, Razdan top, Aharbal, Nilnag, Lolab, Bangus, Reshwar, Margan top, Jawahar tunnel, Ferozpora, Drang, Karnah, Pattan and Duksum (Fig. S1). For the present study, eight natural populations Pissu top (3,286 m asl), Bangus (2866 m asl), Doodhpathri (2,730m asl), Gulmarg (2,657 m asl), Lidderwat (2,634 m asl), Aru (2,417 m asl), Tangmarg (2,158m asl), Dara (2,050 m asl) and one population at Kashmir University Botanical Garden – 1588 m asl (KUBG) (Figure. 1) were selected. The geo-coordinates were taken using Gramin GPS etrex 10. Table 1 depicts the prominent features of the selected sites.

2.3 Morphological Characterization

Twenty mature flowering individuals were randomly selected from the specified populations to observe various morphological parameters. The selected plants were assessed for morphological characteristics such as Rhizome length and breadth, Stem length, basal leaf length and breadth, apical and basal leaf length and breadth, leaf number per plant, number of inflorescences per plant and number of flowers. Populations were selected based on, habitat structure, an abundance of selected plants and ease of access. The specimens from each population were deposited and identified in Kashmir University Herbarium (KASH) under voucher specimens No. 2964, 3749, 4318 and 4319.

Table 1. Habitat features and Geo-coordinates of the selected sites

Study sites	Altitude (m asl)	Latitude (N) and Longitude (E)	Habitat features	Threat
Pissu top	3,286	34°05'02"N 75°26'01"E	Open, dry and rocky slope	Over grazing
Bangus	2866	34°22'14" N 74°02' 45"E	Open, dry and rocky slopes	Overgrazing, habitat destruction
Doodhpathri	2,730	33°51'24" N 74°33'51" E	Open, moist and rocky slope	Overgrazing, habitat destruction
Gulmarg	2,657	34°03'02" N 74°23'14" E	Partial Shady, dry and rocky slope	Local use, Habitat destruction and Overgrazing
Lidderwat	2,634	34°06'32"N 75°14'53" E	Shady slope	Over grazing, habitat destruction
Aru	2,417	34°05'27" N 75°15'54" E	Shady slope, moist	Over grazing, habitat destruction
Tangmarg	2,158	34°03'37" N 74°25'27" E	Partial shady, moist	Habitat destruction
Dara	2,050	34°04'11" N 74°54'09" E	Shady, moist and rocky slopes	Over grazing
KUBG	1,595	34°07'38" N 74°50'12" E	Open field, dry	Nil

**Fig. 1.** Map depicting study sites of *B. amplexicaulis* in Kashmir Himalaya

2.4 Resource Allocation

Ten well-developed and flowering plants from the selected study sites were collected for analysing of resource allocation in different plant parts. The selected plants were further split into stem, leaves, rhizome and inflorescence. Freshly collected specimens were weighed and oven dried at 80 °C for 48 h using an electronic balance [15]. Reproductive effort (RE) was calculated from the evaluation of biomass (dry weight) dedicated to reproductive and above ground vegetative structures [16].

$$RE = \frac{\text{Dry weight of inflorescence}}{\text{total dry weight of the above and below ground parts}} \times 100$$

2.5 Data Analysis

The difference in morphological characters was carried out to test for differences between populations using ANOVA (IBM-SPSS software, version 23). Origin 2021 was used to carry out linear regression between altitude and numerous morphological parameters. Principal component analysis (PCA) was employed to examine morphological features concerning habitat dynamics and comprehend the coherence between various vegetative and reproductive traits.

3. RESULTS

The widespread survey of the Kashmir Himalayas illustrates a wide range of habitats suitable for the growth of *B. amplexicaulis*. The specie grows mainly on rocky slopes (open and partial shady), with an altitudinal range of 1500 m to 3300 m asl (Fig. S2). The morphological features of *B. amplexicaulis* are depicted in Table S1 and also presented in Figure 2. The phenotypic traits of *B. amplexicaulis* studied during the field survey vary substantially, signifying remarkable phenotypic variation between populations across different altitudes (Table 2). The plant height is highest in KUBG (98.54±2.36 cm) and lowest in Pissu top (23.96±3.38 cm). Similarly, Rhizome length and breadth were maximum in KUBG (18.98±1.72 and 2.17±0.31 cm) and minimum in Pissu top (8.66±0.54 and 1.27±0.44 cm). The number of leaves ranged from 3.00±0.67 (Pissu top) to 4.40±0.51 per plant (KUBG). The mean apical and basal leaf length per plant is 4.17±0.72 cm (Pissu top) to 9.62±2.03 cm (KUBG) and

6.51 ± 0.95 cm (Pissu top) to 12.64 ± 2.57 cm (KUBG), respectively. The mean apical and basal leaf breadth per plant ranged from 2.13±0.56 (Pissu top) to 4.57±1.62 (KUBG) and 2.66±0.66 (Pissu top) to 5.38±1.79 cm (KUBG), respectively. Inflorescence length also varied considerably from 2.12±0.43 (Pissu top) to 8.12±0.54 (KUBG) and the number of flowers ranged from 30.4±5.32 (Pissu top) to 95.6±2.99 cm (KUBG) respectively.

The current study demonstrates that resource partitioning is not homogenous amongst different parts of *B. amplexicaulis*. It displayed major differences were displayed in the patterns of resource allocation across the study sites (Table 3). A notable variation was witnessed in the plants across different populations along the altitudinal gradient inhabiting varying habitats with reference to above and belowground dry weight biomass. Maximum resource allocation (dry weight) was exhibited by rhizome (16.92±2.42 to 9.01±1.13 g) followed by the stem (1.85±0.72 to 0.945±0.27 g), leaves (0.98±0.15 to 0.34±0.14 g) and minimum in inflorescence (0.424±0.06 to 0.19±0.08 g). Significant variation was observed in the resource budget per plant of low and high-altitude populations, as values were maximum at low-altitude populations viz. KUBG, Dara and Tangmarg (19.816±3.4, 18.0±4.60 and 16.2±2.30 cm respectively) and minimum at high altitude populations viz. Pissu top, Bangus and Doodhpathri (11.36±1.60, 11.53±3.69 and 12.38±2.32 cm respectively) as represented in Table 3.

Percent resource allocation followed a distinct trend across all populations with maximum resources allocated to rhizome (87.51±3.76 to 78.52±5.17 %) followed by Stem (9.37±3.29 to 7.00±2.50 %), Leaf (8.46±2.49 to 3.99±1.43 %) and inflorescence (3.85±0.70 to 1.00±0.39 %). The reproductive effort also shows a clear trend as plant populations of lower elevations had lesser reproductive effort compared to the populations of higher altitudes. Reproductive effort of populations from Pissu top, Bangus has maximum percent values (20.27± 0.71, 18.99±9.63 % respectively). In comparison to populations from lower altitudes, which include Tangmarg, Dara and KUBG (11.4±4.7, 9.39±3.77 and 6.9±0.56 % respectively).

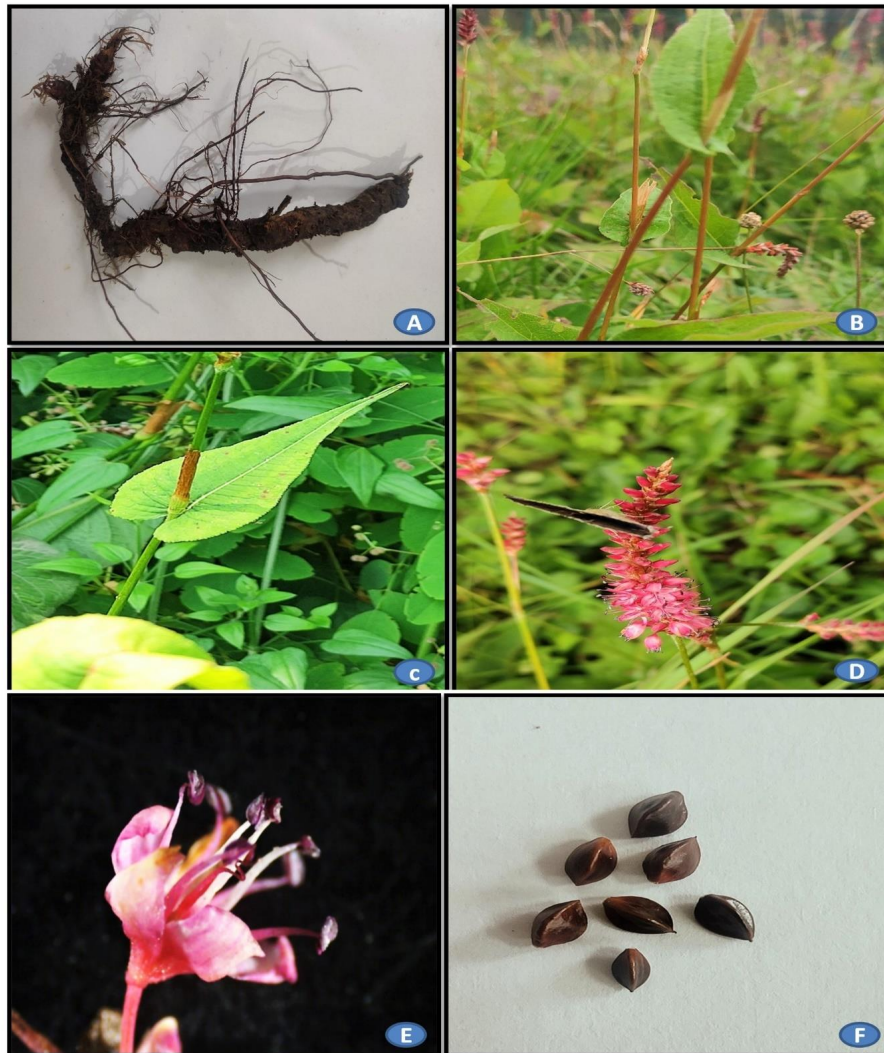


Fig. 2. Morphological features of *Bistorta amplexicaulis* (D. Don) Greene; (A) Rhizome, (B) Stem, (C) Leaf, (D) Inflorescence, (E) Flower, (F) Seeds.

4. DISCUSSION

B. amplexicaulis grows in a wide range of habitats varying from shady slopes, and open plains to rocky and moist slopes from (1585-3300 m asl) in Kashmir Himalaya. Lie *et al.* 2003 [17] indicated a widespread distribution for the species and recorded its distribution in shady grassy places on mountain slopes, grassy slopes in valleys, mountain slopes of forests and on forest margins (1000-3000 m asl). This in turn acquaints us with the fact that the study specie has a broader niche and can show phenotypic plasticity. In the present study we were able to scrutinize significant phenotypic variability and their regression across the populations of different habitats along an elevational gradient (Figure 3). Populations growing at high altitudes (Pissu

top, Bangus and Doodhpathri) are comparatively shorter than the populations of low altitudes (Tangmarg, Dara and KUBG). Thus altitude, plant height and other morphological traits are negatively correlated (Figure 4). r^2 values were calculated where altitude and plant height had $r^2 = 0.9218$. Other morphological traits are positively correlated with plant height. The characters include rhizome length ($r^2 = 0.9338$), rhizome breadth ($r^2 = 0.9108$), apical leaf length ($r^2 = 0.9718$), apical leaf breadth ($r^2 = 0.9951$), basal leaf length ($r^2 = 0.9118$), basal leaf breadth ($r^2 = 0.6718$), inflorescence length ($r^2 = 0.9318$). PCA (Figure 5) has shown that high altitudes are disparaging for almost all of the reproductive and vegetative traits of *B. amplexicaulis* demonstrating superior growth conditions at lower altitudes [18]. Increasing altitude causes plants

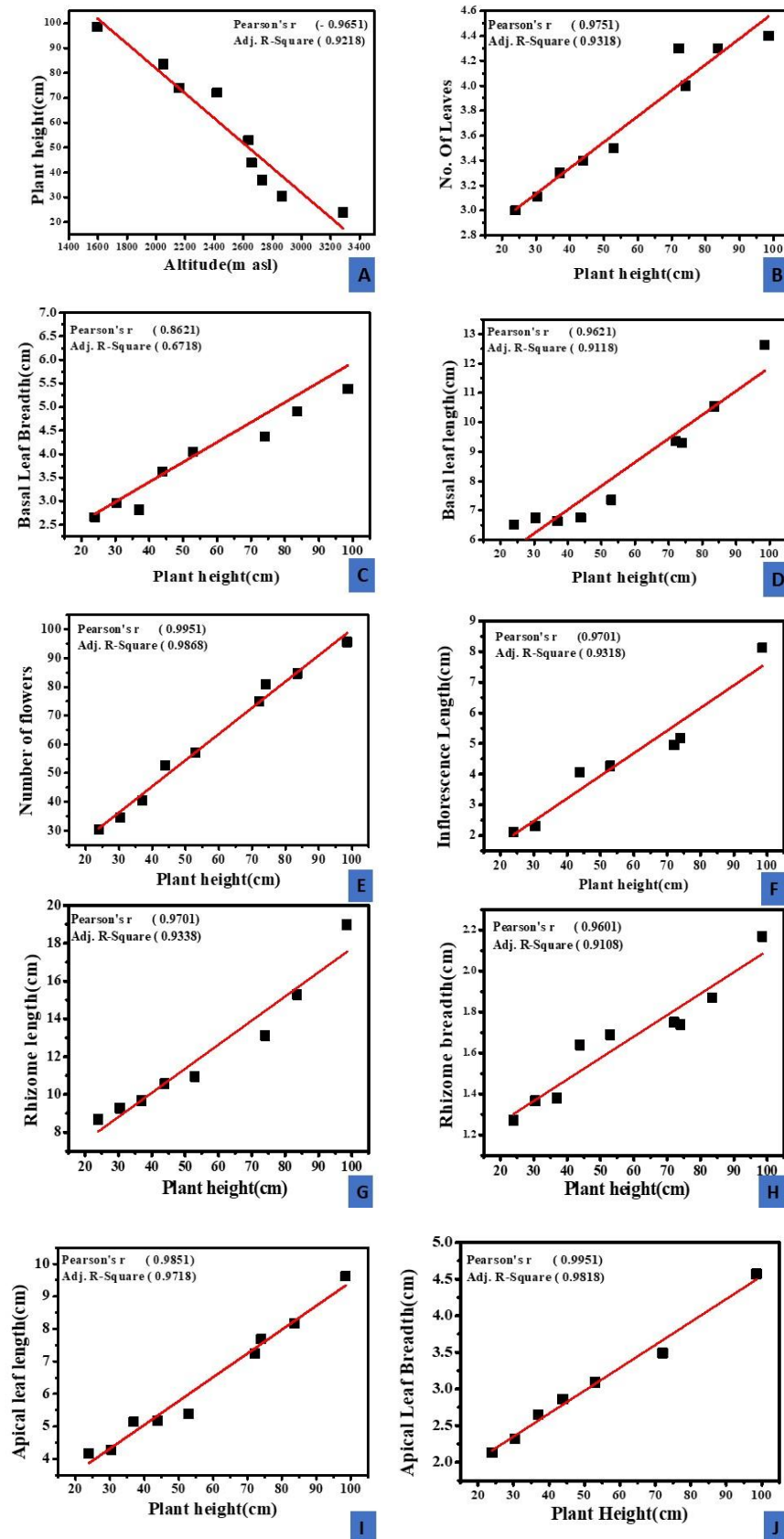


Fig. 3. (A-J) Regression analysis of various morphological traits of *Bistorta amplexicaulis*

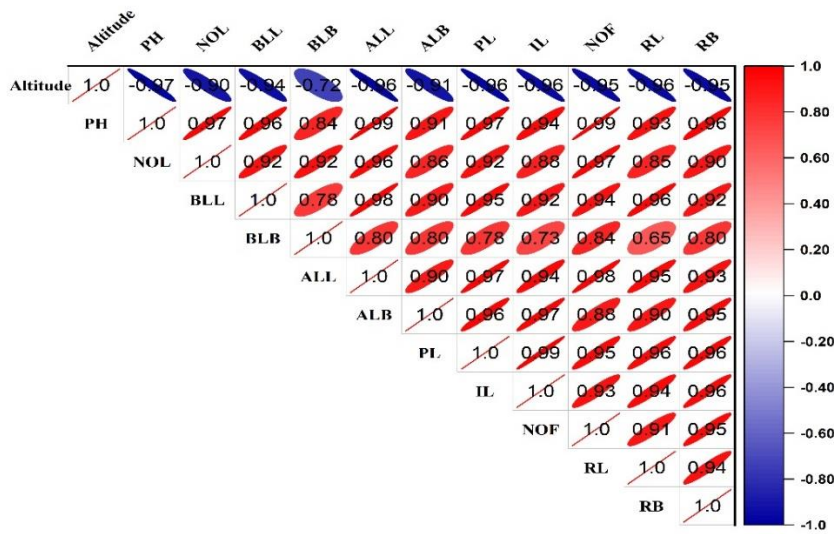


Fig. 4. Correlation plot showing Pearson's correlation coefficient amongst altitude and various morphological traits. PH. Plant height; NOL. Number of leaves; ALL. Apical leaf length; ALB. Apical leaf breadth; BLL. Basal leaf length BLB. Basal leaf breadth; IL Inflorescence length; NOF. Number of flowers; RL. Rhizome length; RB. Rhizome breadth

to grow shorter which can be a survival mechanism to endure harsh climatic conditions such as strong winds, also as leaves remain close to the warmer soil, photosynthetic conditions are amended [19]. At higher altitudes plants intensify super cooling capability by diminishing intercellular spaces and cell size [20]. Which ultimately results in an overall decrease in plant size. This reverse relationship between plant height and increasing altitude, as an adaptation has already been reported by various workers [21-24]. Apart from the severe conditions, plants growing at high altitudes have shorter growing seasons, which are less tall compared to plants at lower altitudes, where the growth period is relatively longer [25, 26].

The most important components of a plant's photosynthetic system are its leaves, which are essential for both plant functioning and enduring environmental adaptability [27]. Essential leaf traits include leaf shape, biomass, and water content [28]. As leaf dry matter content shows the balance between investment in storage and growth, it has frequently been used to forecast growth strategy and responsiveness to environmental perturbations. According to this study, the leaf length and breadth were found to be greatest in plant populations at lower altitudes (Dara, KUBG) and minimum in plants

at higher altitudes (Pissu top, Bangus). According to Bresson *et al.* 2011 [29], increasing altitude generally causes a decrease in the length, breadth, and area of leaves. Light is a vitally important limiting factor for plant development and survival at low altitudes [30]. Due to their restricted capacity for photosynthetic activity per unit leaf area, plants that grow in low light intensities would naturally allocate the majority of their biomass to laminae [31, 32]. Large leaves can intercept a significant quantity of light at low light intensities because of their larger foliar display [33].

Understanding the patterns of resource allocation is important for understanding the life history strategies of different plant species. In this study species, allocation of most of the resources is towards the rhizome. After rhizomes, most resources get allocated to the stem followed by leaf and inflorescence across all populations at different altitudes. Even though the relative percent resource allocation follows a similar trend, there is a significant difference in the dry weight biomass amongst the populations at different altitudes. At higher altitudes, relatively more dry mass is accumulated in comparison to low altitude plants. In populations of Pissu top and Bangus the ratio of Dry weight/ fresh weight for rhizomes is 0.616 g and whereas for Dara and KUBG it is

Table 2. Morphological features and their variation (mean \pm SD) across different study sites of *B. amplexicaulis*

*Means labelled with the small letters designate that they vary significantly from each other amongst the selected populations (Tukey test: $P \leq 0.05$)

*Mean \pm Standard Error

Phenotypic traits	Pissu top	Bangus	Doodhpathri	Gulmarg	Lidderwat	Aru	Tangmarg	Dara	KUBG	F valve
Plant height (cm)	*23.96 \pm 3.38 ^a	30.433 \pm 1.12 ^b	36.93 \pm 2.84 ^c	43.89 \pm 2.27 ^d	52.29 \pm 2.06 ^e	72.08 \pm 1.59 ^f	74.03 \pm 2.38 ^f	83.58 \pm 2.69 ^g	98.54 \pm 2.36 ^h	24.938
No. of leaves per plant	3 \pm 0.67 ^a	3.11 \pm 0.78 ^b	3.3 \pm 0.67 ^{ab}	3.4 \pm 0.84 ^{abc}	3.5 \pm 0.52 ^{abc}	4.3 \pm 0.48 ^c	4.00 \pm 0.82 ^{bc}	4.3 \pm 0.57 ^{bc}	4.4 \pm 0.51 ^c	0.907
Basal leaf length (cm)	6.51 \pm 0.95 ^a	6.744 \pm 1.47 ^a	6.636 \pm 2.20 ^a	6.755 \pm 2.90 ^a	7.36 \pm 2.52 ^{ab}	9.37 \pm 2.62 ^{ab}	9.31 \pm 2.16 ^{ab}	10.54 \pm 1.32 ^{bc}	12.64 \pm 2.57 ^c	1.784
Basal leaf breadth (cm)	2.66 \pm 0.66 ^a	2.95 \pm 1.11 ^a	2.8165 \pm 1.00 ^a	3.62 \pm 1.03 ^{ab}	4.05 \pm 1.12 ^{abc}	6.51 \pm 2.15 ^c	4.37 \pm 0.65 ^{abc}	4.91 \pm 0.731 ^{bc}	5.38 \pm 1.79 ^c	4.557
Apical leaf length (cm)	4.17 \pm 0.72 ^a	4.27 \pm 1.10 ^a	5.14 \pm 1.09 ^{ab}	5.18 \pm 1.11 ^{ab}	5.39 \pm 1.76 ^{ab}	7.24 \pm 2.39 ^{bc}	7.69 \pm 2.13 ^{cd}	8.17 \pm 0.97 ^{cd}	9.62 \pm 2.03 ^d	1.201
Apical leaf breadth (cm)	2.13 \pm 0.56 ^a	2.32 \pm 0.69 ^a	2.65 \pm 0.64 ^a	2.86 \pm 1.01 ^a	3.09 \pm 1.18 ^a	3.49 \pm 1.22 ^{ab}	3.05 \pm 0.55 ^a	3.22 \pm 0.52 ^{ab}	4.57 \pm 1.62 ^b	1.393
Inflorescence length (cm)	2.12 \pm 0.43 ^a	2.31 \pm 0.38 ^a	3.88 \pm 0.51 ^b	4.07 \pm 0.70 ^b	4.27 \pm 0.75 ^{bc}	4.96 \pm 0.49 ^{cd}	5.18 \pm 0.89 ^d	5.35 \pm 0.37 ^d	8.12 \pm 0.54 ^e	13.661
No. of flowers	30.4 \pm 5.32 ^a	34.44 \pm 3.94 ^a	40.50 \pm 3.72 ^b	52.70 \pm 3.5 ^c	57.00 \pm 4.61 ^c	75.00 \pm 3.33 ^d	80.8 \pm 3.33 ^e	84.6 \pm 2.4 ^e	95.6 \pm 2.99 ^f	8.705
Rhizome length (cm)	8.66 \pm 0.54 ^a	9.27 \pm 1.39 ^{ab}	9.69 \pm 0.54 ^{abc}	10.56 \pm 1.12 ^{abc}	10.96 \pm 0.88 ^{bc}	11.46 \pm 0.95 ^{cd}	13.12 \pm 2.21 ^d	15.99 \pm 0.73 ^e	18.98 \pm 1.72 ^f	9.497
Rhizome breadth (cm)	1.27 \pm 0.44 ^a	1.36 \pm 0.15 ^{ab}	1.38 \pm 0.46 ^{ab}	1.64 \pm 0.28 ^{abc}	1.69 \pm 0.58 ^{abc}	1.75 \pm 0.36 ^{abc}	1.74 \pm 0.16 ^{abc}	1.87 \pm 0.221 ^{bc}	2.17 \pm 0.31 ^c	0.5

Table 3. Allocation of resources (mean \pm SD) towards different plant parts in *B. amplexicaulis* across different study sites.

Traits	Pissutop	Bangus	Dodhpathri	Gulmarg	Lidderwat	Aru	Tangmarg	Dara	KUBG	F value
Rhizome (g)	*9.012 \pm	9.19 \pm	10.23 \pm	11.30 \pm	12.15 \pm	12.88 \pm	14.15 \pm	15.40 \pm	16.92 \pm	21.009
	1.13 ^a	2.95 ^a	1.27 ^{ab}	2.19 ^{ab}	1.88 ^{bc}	1.41 ^{bcd}	1.06 ^{cd}	1.36 ^{de}	2.42 ^e	
Stem (g)	0.945 \pm	0.97 \pm	1.04 \pm	1.05 \pm	1.083 \pm	1.15 \pm	1.161 \pm	1.78 \pm	1.85 \pm	2.951
	0.27 ^a	0.22 ^a	0.51 ^a	0.15 ^a	0.47 ^a	0.45 ^a	0.50 ^a	2.95 ^a	0.72 ^a	
Leaf (g)	0.98 \pm 0.15 ^a	0.91 \pm	0.76 \pm	0.60 \pm	0.61 \pm	0.68 \pm	0.65 \pm	0.7 \pm	0.83 \pm	1.029
		0.29 ^a	0.37 ^a	0.33 ^a	0.12 ^a	0.25 ^a	0.28 ^a	0.20 ^a	0.18 ^a	
Inflorescence (g)	0.424 \pm	0.44 \pm	0.34 \pm	0.32 \pm	0.33 \pm	0.30 \pm	0.247 \pm	0.19 \pm	0.197 \pm	1.584
	0.06 ^{ab}	0.24 ^b	0.18 ^{ab}	0.10 ^{ab}	0.21 ^{ab}	0.20 ^{ab}	0.19 ^{ab}	0.08 ^a	0.08 ^a	
Total resource budget per plant (g)	11.13 \pm	11.53 \pm	12.38 \pm	13.28 \pm	14.19 \pm	15.02 \pm	16.21 \pm	18.07 \pm	19.81 \pm	4.905
	1.23 ^a	3.00 ^a	1.74 ^{ab}	2.06 ^{abc}	2.31 ^{abc}	1.56 ^{bcd}	1.59 ^{cd}	3.85 ^d	2.65 ^e	
Reproductive effort (%)	20.27 \pm	18.99 \pm	17.17 \pm	16.28 \pm	15.78 \pm	14.20 \pm	11.4 \pm	9.39 \pm	6.9 \pm	15.288
	0.71 ^c	9.63 ^c	7.31 ^{bc}	4.33 ^{bc}	7.81 ^{abc}	7.91 ^{abc}	4.7 ^{abc}	3.77 ^{ab}	2.56 ^a	

* Means labelled with the small letters designate that they vary significantly from each other amongst the selected populations (Tukey test: $P \leq 0.05$)*Mean \pm Standard Error

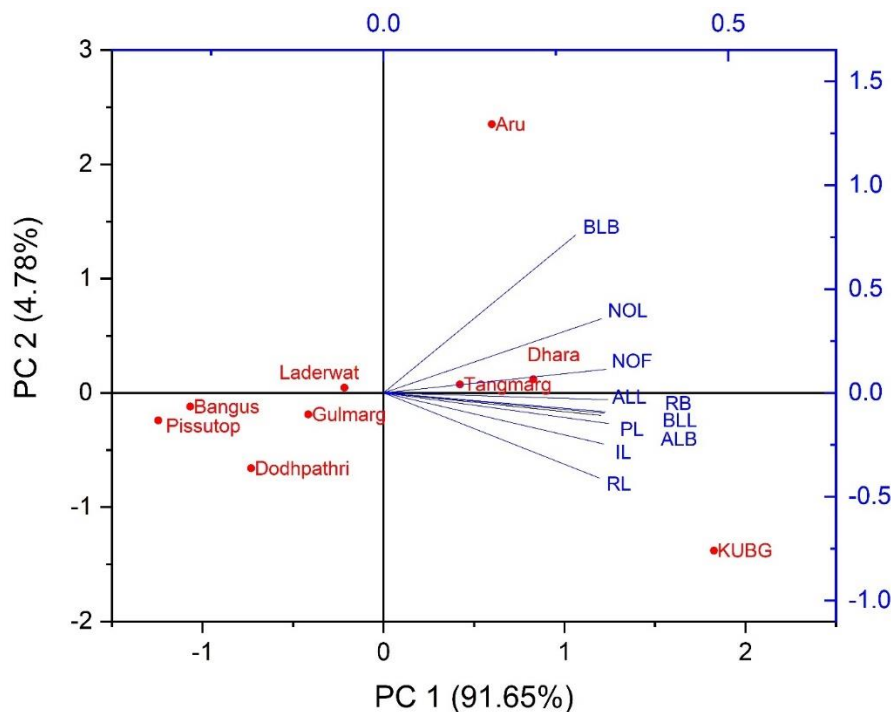


Fig. 5. Principal Component Analysis (PCA) plot of various morphological features of *Bistorta amplexicaulis* across the different study sites.

0.390 g and 0.388 g respectively. A similar trend is followed for stem, leaf and inflorescence. Reproductive effort decreased with decreasing altitude, this is in accordance with various some previous studies [18, 34]. It is also reported that with the increase in plant size, reproductive effort declines [35, 36]. The number of flowers per plant varies significantly ($P \leq 0.05$) along the altitudinal gradient, with lower altitudes having the maximum flowers per plant (Dara, KUBG). In energy-limited environments where survival is the primary concern, resource allocation towards vegetative organs is critical rather than spending resources for the reproductive purposes [37]. The findings support Johnson and Cook's [38] who stated that plants at higher altitudes have lesser flowers than plants growing at lower altitudes. It has also been documented that sexual reproduction in alpine locations is generally low when compared with that of the warmer regions [39].

5. CONCLUSION

It is concluded that environmental heterogeneity is the leading cause of phenotypic variation in *B. amplexicaulis*. Our findings demonstrated a diverse variety of favourable environments for the growth of *B. amplexicaulis*. Along an altitudinal gradient, the specie exhibits considerable variability in morphological

traits such as stem height, inflorescence length, and leaf and rhizome dimensions. Most of the characters demonstrated a negative correlation with increasing altitude. The most prominent effects were recorded for stem height and leaf dimensions. Given that low altitude population experience less environmental stress, it may be concluded from the current study that these populations were substantially more robust in terms of their morphological characteristics and thus these sites are more suitable for the growth and development of this particular medicinal plant. The total budget (dry weight) showed a negative correlation with increasing altitudes. Further studies related to the effect of other environmental variables such as physicochemical properties of soil and climate (temperature, precipitation and rainfall) would help us to understand if there are any other factors responsible for the phenotypic variability across different altitudes.

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7. CONFLICT OF INTEREST

The authors declare that they have no known competing

financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY DATA

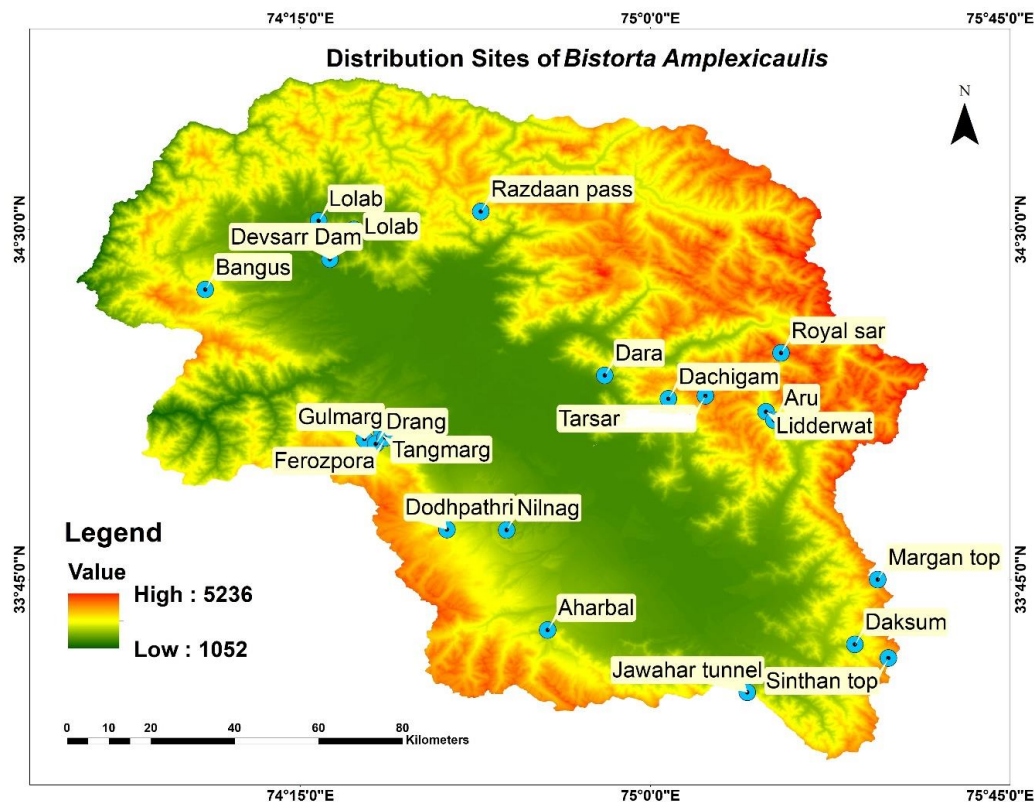


Fig. S1. Map showing distribution sites of *B. amplexicaulis* in Kashmir Himalaya



Fig. S2. Comparative features of different habitats of *B. amplexicaulis* in Kashmir Himalaya (A) Open, dry and rocky slope (Pissu top) (B) Open, moist and rocky slope (Doodhpathri) (C) Shady slope, moist (Aru) (D) Partial shady, dry and rocky slope (Gulmarg) (E) Partial shady and moist (Tangmarg) (F) Open dry and rocky slopes (Bangus)

Table S1. Different morphological features of *B. amplexicaulis*

Habit	Herbaceous, Perennial
Root	Thick rhizome, with attached fibrous roots.
Stem	Erect 20 - 100 cm, circular in outline
Leaf	Petiolate, lanceolate, Opposite, basal leaves larger (6- 12cm) then apical leaves (4-10 cm), tip acuminate. Leaves are peculiarly stem-clasping (amplexicaule), stipules sheathing giving the stem a jointed appearance at each leaf node.
Inflorescence	Spikes are stalked (2- 9 cm long) rose-red or pinkish in color, narrow, pencil-thin, long-stalked spikes. Flowers are bisexual
Tepals	5, pinkish in colour, proximally connate
Androecium	8 stamens, 5 + 3 arrangement, free, bilobed anthers.
Gynoecium	The ovary consists of three united carpels that form a single locule, which produces only one ovule, ovary superior, basal placentation Styles 3, free; stigmas 3
Fruit	Achene



Quality Assessment of Fried Oils from Different Street Food Vendors and Restaurants in Different Areas of Gilgit, Pakistan

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Abstract: The study was designed to estimate the quality of the frying oils used in northern areas of Pakistan. A cross-sectional analysis was performed to investigate the behavior and common practices of street food vendors (SFVs) regarding oil selection, food preparation, and awareness of rancid oil on human health. Seventy-Eight (78) commercial fried oil samples were evaluated based on the free fatty acid (FFAs), peroxide value (PV), moisture contents (MC), total polar matter (TPM), color, and iodine value (IV). The analysis showed that FFAs, PV, TPM, color, and IV significantly deviated from standard values provided by Pakistan Standard Quality Control Authority, (PSQCA) Pakistan. The SFVs used low-quality oil because of low price and ease of availability over quality, frying oil was changed infrequently and blended with new oil. Furthermore, the majority of SFVs were unaware of the hazards of rancid oil to human health, food handling practices were unsanitary, and cleaning methods were ineffective. Quality control, legislation, and SFVs safety and hygiene training are the most critical requirements to improve the overall quality of fried street foods in Gilgit, Pakistan.

Keywords: Street Food Vendors, Quality, Deep frying, Rancidity, Pakistan

1. INTRODUCTION

Street foods (SF) come under the category of food or drink sold by vendors, hawkers, and small stalls located in a street, local markets, and any other public places that can be consumed instantaneously. SF is more in demand due to its cheap prices, easy availability, and being culturally accepted either in rural or urban areas. Past studies have revealed that about 2.5 billion people are involved in the consumption of SF globally [1, 2].

People purchased SF normally from street food vendors (SFVs) including mobile SFVs, fixed stall SFVs, and semi-fixed SFVs. The working conditions of SFVs have normally been characterized as low incomes, absence of social security or state benefits, extensive working hours, and unsafe working environments [3]. The literacy rate among SFVs is found to be very low and they are observed as untrained in maintaining food safety and hygiene

[4]. The ignorance of SFVs in following the food safety regulations not only compromises the quality of food but is also associated with the outbreak of major food-borne illnesses [5].

In Pakistan, people preferred to choose ready-to-eat foods available at low prices due to their low income [6]. In the past few years, major cities of Pakistan have suffered a significant increase in urbanization mainly due to the high rate of migration from rural areas. The pressure of increased population in urban areas tends to cause SF vending businesses to grow at an extensive rate. SFVs earn their livelihood by catering to the needs of a large number of migrant workers far away from their home places [7].

People preferred to fill their snack cravings with foods sold by SFVs available nearby their location. SFVs usually compromised the quality of fried foods by using cheaper substitutes for frying

oil [4]. The replacement of cheaper oil substitutes, use of low-quality utensils, and improper cleaning methods lead to a highly contaminated poor quality end product that adversely affects consumer health [8]. SFVs associated with selling fried food items mainly target crowded areas such as bus stands, commercial areas, and educational and industrial zones to capture more people [7]. Most of the vendors preferred to reuse the leftover oil over and over without being properly stored. Moreover, SFVs are not well aware of personal hygiene and do not follow cleaning and safety practices. The poor infrastructure, improper sanitation, and poor personal hygienic practices are directly associated with the proliferation of microbial hazards, chemical contamination, and environmental pollution [9-10]. SFVs mostly use locally manufactured unhealthy fat obtained from animal sources, the frequent consumption of unhealthy fats causes cardiovascular diseases and carcinogenic impacts on human health [11].

The relatively high temperature (150-180 °C) and repeated heating of cooking oil affect the quality by increasing the viscosity, darkening of color, and nutritional changes by altering the fatty acid profile due to hydrolysis, oxidation, thermal polymerization and pyrolytic reactions [12]. Consequently, various by-products including esters, aldehydes, ketones, and peroxides can be produced and absorbed by fried foods [13]. The severity of chemical changes during frying reactions is dependent on duration, method of heat treatment, frying medium, and type of product [14]. A study reported that the fifty repeated heating of sunflower oil (160-200 °C) for 5 mins on the same day produced low-quality end products [15]. This discarding point can be easily detectable by physical indicators, the oil must be discarded or changed, when it turns dark, produces excessive smoke and a strong odor, and cause the greased texture of the product [13]. Another study also reported that change in the physicochemical properties of the frying oil samples collected from Multan, Pakistan. The results of the study showed that FFA, conjugated dienes, and heavy metals concentrations (cobalt and nickel) were normal, while, peroxide values, Cd, and Pb were more than normal [16]. A recent study conducted in Lahore, Pakistan also reported the quality of fifty frying oil samples being used by SFVs. The results of the study reported that most of the frying oil samples

were unhealthy and exhibited higher moisture content ($> 0.10\%$), total polar content ($> 25\%$), peroxide value (>10 meq.O₂/kg), and free fatty acid (> 0.20 mg KOH/g); and very low IV (< 80 g/100 g) [17]. Another similar study conducted in Faisalabad, Pakistan also reported that fifty oil samples were collected and most of them were found oxidized and degraded [18].

Considering the importance of the literature cited above, the present study was first time conducted in Gilgit (a crowded city in the northern area) of Pakistan to evaluate the knowledge, selection, and oil preferences for frying by the SFVs. The quality of oils used for frying was also analyzed using a set of physicochemical attributes including moisture content (MC), total polar matter (TPM), color determination, free fatty acid (FFAs), and peroxide value (PV), and compared the results with the standard values reported by the PSQCA, Pakistan. Moreover, this study also determined the ratio of rancidity among the oil samples used by SFVs in northern areas particularly Gilgit, Pakistan.

2. MATERIALS AND METHODS

2.1 Sample and Data Collection

In this cross-sectional study, a total of 78 frying oil samples were collected from different SFVs and restaurants present in eight different areas of Gilgit, Pakistan including Ghizer, Sonikot, NLI market, Danyor, Kashrot, bus stand Gilgit, Jutial, and Konodas. The homogeneous samples (approx. 700 mL) were collected in pre-cleaned transparent containers. Collected samples were coded and transported for analysis in dark boxes with ice sheets. Later, the oil samples were filtered and stored at 4 °C for further analysis. To assess the status of frying oil and its handling, a survey questionnaire was compiled to record feedback from SFVs and restaurant workers. The parameters discussed in the questionnaire included the frequency of oil change, hygiene habits of SFVs, awareness regarding rancidity and its impact on human health, and preferences regarding the selection of oil.

2.2 Qualitative Assessment of Fried Oils

For qualitative assessment of fried oils following physicochemical parameters were performed. The tests were performed based on the quality control

parameters set by the PSQCA, Pakistan.

2.2.1 Total polar matter

The TPM was analyzed by following the procedure reported by Ghobadi *et al.* [19] using the TPM digital counter (VITO®, Tuttlingen, Germany). The sensor of the TPM counter was placed in heated oil samples (120 °C) and the reading was noted after 30 sec. The sensor indicated the quality of the oil by showing the color; the presence of green color indicated that the quality of the oil is not degraded yet; while the red color indicated the oil has been degraded. Further, the concentration of TPM was determined by the value that appear on the probe.

2.2.2 Moisture content

The MC of the samples was measured by following the method by AOAC: 2005 [20]. The MC in oil samples was determined through drying in a hot air oven at 105 °C. About 50 g of each oil sample was taken into a pre-cleaned and pre-weighed petri dish. The drying was performed for 6 h at 105 °C until the constant weight gain. The difference in weight was calculated by using the formula (1)

$$\text{Moisture content (\%)} = \frac{W_1 - W_2}{W_1} \times 100 \quad (1)$$

Where;

W_1 = weight(g) of the sample before drying

W_2 = Weight(g) of the sample after drying

2.2.3 Color determination

The 20 g sample of each fried oil at a temperature of 50 °C was placed in a transparent petri dish. The digital colorimeter (Chroma Meter, Konica Minolta, Japan) was used to determine the color of the frying oil samples. The sensor of the colorimeter was positioned directly on the light path to measure the R-value and Y-value and compared them with the standard value of 5.0 and 50, respectively, set by the Codex Alimentarius maximum level [21].

2.2.4 Free fatty acids

The production of FFAs was determined by following the method reported by Folayan *et al.* [22] with slight modifications. Pure ethanol (20 mL of 95 %) was added to 10 g of each fried oil sample.

A few drops of phenolphthalein as an indicator were added and the mixture was titrated with 0.1 N NaOH. The amount of FFAs in terms of oleic acid oil can be expressed by the formula (2).

$$\text{FFA (\%)} = \frac{V \times N \times 28.20}{\text{Sample weight (g)}} \quad (2)$$

Where;

V = volume of NaOH used (mL)

N = normality of NaOH used

2.2.5 Iodine value

The IV of oil samples was determined following the method of ISO 3961:2009 [23] with slight modification. 0.2 g of oil was dissolved with 20 mL of chloroform the resultant mixture was further mixed with 20 mL of iodine solution and placed in the dark for 30 mins. After that 15 % of potassium iodide (KI) was added to 100 mL of distilled water and mixed with the aforementioned mixture and titrated with 0.1 N of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$). Starch was added as an indicator and titrated until transparent. The IV was determined by the amount of iodine absorbed by 100 g of the oil.

2.2.6 Peroxide value

PV of the oil samples was performed by the following method of ISO 3960: 2007 [24] with slight modification. The 5 g of each oil sample was poured into a 250 mL Erlenmeyer flask then the 25 mL mixture of acetic acid and chloroform was added in the ratio of (3:2). After proper mixing, about 0.5 mL KI was added and mixed for 1 min. Afterward, 35 mL of distilled water was added to the same Erlenmeyer flask. The standard starch solution (0.5 mL) was added as an indicator and kept for a half hour in the dark. Then, the mixture was titrated with 0.01 N ($\text{Na}_2\text{S}_2\text{O}_3$) solution until the blue color turned transparent. PV can be calculated by using the formula (3).

$$\text{PV} = \frac{(S-B) \times N \times 1000}{\text{Sample weight (g)}} \quad (3)$$

Where,

S = burette reading of 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$ with sample

B = burette reading of 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$ without sample

N = normality of $\text{Na}_2\text{S}_2\text{O}_3$ solution used

2.3 Statistical Analysis

The data analysis was performed on SPSS version 21. The obtained results were analyzed using the z-test method along with their mean value and standard deviation. All the experiments were performed in triplicates. Moreover, the reliability of the survey was analyzed using Cronbach's alpha as the standard value.

3. RESULTS AND DISCUSSION

3.1 Survey-based Analysis by SFVs

A survey questionnaire was designed to evaluate the knowledge, selection, and preferences of SFVs for the oil used during frying. The Cronbach's alpha value with a coefficient of 0.895 indicated that the survey was reliable and can predict the impact of evaluated questions. The parameters studied in the questionnaire include oil quality, mixing of the oil with solid fats, less frequent oil changes, unsanitary methods, and ineffective cleaning methods. According to the results of the survey, the quality of frying oil samples was found poor and SFVs have no information regarding safety practices. This could be due to a lack of knowledge about frying methods and safety practices associated with the oil used for the frying. A full overview of the variables that were discussed in the questionnaire along with the absolute and relative frequency is presented in Table 1.

Table 1 presents that 93.59 % of SFVs preferred to use cooking oil for deep frying of foods and among them, 65.38 % preferred to use sunflower oil as a frying medium, while 6.41 % of SFVs preferred animal fat for frying. Their preference for choosing cooking oil was based on ease of availability and access. About 42.31 % of SFVs tend to add the new oil to used oil to increase the quantity, while 33.33 % of SFVs completely discarded the used oil, the highest trend was observed in Ghizer (11.54 %) followed by Jutial (5.12 %). About 24.36 % of SFVs continued to use the same oil until it cannot be further used. A study is in strong agreement with the current study reported that over the globe, 75 % of plant-based oil (corn oil, sunflower oil, and olive oil) consumption is for the preparation of snack food, frying oils, shortening, and salad dressings [25].

It was also observed that the frequency of frying oil change was mainly dependent on the sale of fried products. If demand increased, the frequency of oil changes increased, otherwise, they used to change it every single day or two days. Table 1 showed that 66.67 % of SFVs changed the oil on daily basis, and 29.49 % SFVs used the same oil for more than 2 days. This practice was performed due to a lack of knowledge regarding the health issues associated with the use of repeatedly fried oil. While 3.85 % of SFVs changed the oil more than twice a day and the highest trend was observed in the NLI market (2.56 %). A supported study conducted in India also presented that almost 25 % of SFVs tend to change their oil regularly while 75 % of SFVs did not change their oil daily or used oil without changing it [26]. Another study is in strong agreement with the current study reporting that SFVs in Ethiopia tend to use recycled frying oil on regular basis. This study also extended that recycled or repeated frying oil (at least five times) significantly increased the body weight of rats during the cell line studies [27].

Colour plays important role in determining the physical quality of oil used for frying. About 72 % of SFVs fully utilized the oil daily without considering the change in color of oil; where the highest trend was observed in Ghizer (30.76 %), followed by Danyor (10.25 %), NLI market (8.97 %), Sonikot (7.69 %), Konodas and Kashrot (6.41 % each) and Jutial (1.28 %). While the remaining 28 % of SFVs used the oil until its appearance turned fully blackish.

The behavior of SFVs towards the disposal of used cooking oil showed that 62.82 % of SFVs disposed of used oil in open drains. This practice was mostly observed in Ghizer, Sonikot, NLI market, Danyor, Kashrot, bus stand Gilgit Jutial, and Konodas. About 26.92 % of SFVs discarded the used oil in open fields and 14.10 % of SFVs disposed of the used oil in a separate container. A similar study in Harare, Zimbabwe reported that 93 % SFVs used to discard the waste food in waste bins, while 7 % discarded all the leftovers and waste food in unspecified places on regular basis [28].

The results related to the knowledge of SFVs regarding the rancidity of oil showed that 51.28 % of SFVs found with no knowledge of the rancidity of frying oil, which highest trend was observed in the NLI market (11.53 %) followed by Ghizer

(8.97 %), Danyor (7.69 %), Kashrot and Jutial (6.41 % each), bus stand Gilgit and Sonikot (3.84 %) and Konodas (2.56 %). About 37 % of total SFVs had little knowledge about the impact of rancid oil on human health and it was found that only 1.28 % SFVs were fully aware of the impact of consuming rancid oil on human health.

The trend of using PPE while working was also observed among SFVs. The results showed that 83.33 % SFVs were not using any kind of PPE during frying practices. Only 12.82 % SFVs responded that they have easy access to PPE and were using it as per the requirement. Among them, 3.85% of SFV responded that they have easy access to PPE but never use them because they found the use of PPE uncomfortable while frying. Table 1 showed that 61.54 % have little knowledge about hygiene practices, and only 25.64 % of people were fully aware of personal hygiene and safe handling, while 12.82 % SFVs were found completely unaware. The current study was in agreement with a study conducted in Lucknow, India, where 94 % of the SFVs practiced selling their foods without wearing gloves, and 96 % of the SFVs don't wear headcovers [26]. Another study is in support of the current study, which reported that due to improper safety knowledge, SFVs used to prepare their foods in explicitly unhygienic conditions [29].

The results for the usage of new oil for different food products are also present in Table 1. About 85.90 % SFVs do not prefer to use new oil for frying of variety of food products instead of it they mixed each frying product in the same oil and 8.97 % of SFVs change oil for different food products regularly. Table 1 also showed that 61.54 % of SFVs preferred to use LPG as their source of ignition. While 38.46% SFVs preferred to use wood for ignition and the highest trend was observed in Ghizer (29.44 %) followed by Kashrot (3.84 %), Sonikot, and Jutial (2.56 %). The trend of using vegetable oil instead of animal fat was also observed and 93.59 % were found associated with it. While 6.41 % of SFVs preferred to use animal fat for frying because they considered it a good source for taste enhancement. A study also supported the current results, which presented that restaurant workers and roadside SFVs of Okota, Lagos State Nigeria, preferred to use plant oils. This study further presents that total phenolic content, vitamin E, and vitamin A contents were found significantly

lower in oil samples collected from roadside SFVs and fast food restaurants as compared to the unused oil [30].

3.2 Qualitative and Physicochemical Analysis of Frying Oil Samples

3.2.1 TPM of fried oil samples

The mean TPM values of different fried oil samples are presented in Figure 1. The development of TPM is a predominant indicator to estimate the deterioration in frying oil. The repeated heating frequency induces chemical changes in oils including hydrolysis, oxidation, polymerization, and isomerization [14]. After determining the TPM of all oil samples, the values obtained were in ranged from 17.17 ± 4.28 to 23.36 ± 2.45 . The TPM results presented in (Figure 1) show that the values were significantly higher ($p = 0.00$) than the standard values set by PSQCA (< 18 %). Out of 78 samples, only 4 samples (5.12 %) from Sonikot 17.17 ± 4.28 were under the PSQCA limits and considered fine quality and 74 samples (94.87 %) were not fulfilling the PSQCA standard criteria.

It was concluded that with the increased frying time and high-temperature exposure, the TPM of oil samples get increased. A similar study also presented the significant correlation between the TPM and frying, with the overheating of oil at high temperatures more polar matter is produced due to the heat decomposition of chemical substances [31].

3.2.2 MC of fried oil samples

The mean MC values of different fried oil samples are presented in (Figure 2). MC values obtained from different fried oils samples ranged from 0.08 ± 0.05 to 0.24 ± 0.08 . The values indicated that most of the oil samples for MC were under the limit as set by the PSQCA (0.15 %) except for the three samples that were deviating from the standard limits. Overall, there was a non-significant relation ($p = 0.51$) observed among the MC of frying oil samples. The highest value was reported in the NLI market at 0.24 ± 0.08 followed by Gilgit bus stand 0.2 ± 0.10 , Kashrot 0.16 ± 0.08 , Konodas 0.118 ± 0.116 , Jutial 0.11 ± 0.13 , Danyor 0.10 ± 0.11 , Sonikot 0.101 ± 0.07 and Ghakuch 0.08 ± 0.05 . Possible reasons for higher moisture content

Table 1. Results of survey-based analysis of fried oil samples collected from restaurants and SFVs in Gilgit, Pakistan (N =78)

Variables	Absolute frequency (N)	Relative frequency (%)
<i>Preference for frying</i>		
Cooking oil	73	93.59
Animal fat	5	6.41
Any Other	0	0.00
<i>Reason of preference</i>		
Quality	51	65.38
Availability	16	20.51
Price	11	14.10
<i>Utility of oil per day</i>		
Dispose of it	26	33.33
Mix it in new oil	33	42.31
Continuously use it	19	24.36
<i>Frequency of oil change</i>		
2 or more times a day	3	3.85
Daily	52	66.67
More than 2 days	23	29.49
<i>Physical parameters use to change the oil</i>		
Blackish appearance	22	28
Fully utilization	56	72
Other	0	0
<i>Where used oil was disposed</i>		
Drain	49	62.82
Separate container	11	14.10
In open field	21	26.92
<i>Knowledge of rancid oil's impact on human health</i>		
Yes	1	1.28
Little knowledge	37	47.44
No	40	51.28
<i>Trend of using personal protective equipment (PPE)</i>		
Available and use	10	12.82
Not available	65	83.33
Available but don't use	3	3.85
<i>Knowledge about personal hygiene and handling</i>		
Fully aware	20	25.64
Somehow	48	61.54
Not at all	10	12.82
<i>Usage of oil for different items</i>		
Yes	4	5.13
Sometime	7	8.97
No	67	85.90
<i>Medium of heating</i>		

Variables	Absolute frequency (N)	Relative frequency (%)
Wood	30	38.46
LPG	48	61.54
Kerosene oil stove	0	0.00
<i>Usage of animal fat for frying</i>		
Yes	5	6.41
Somehow	0	0.00
No	73	93.59

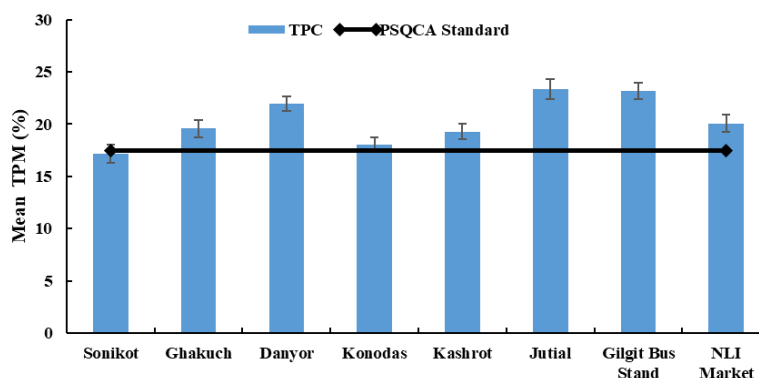


Fig. 1. Mean TPM obtained from oil samples from different SFVs.

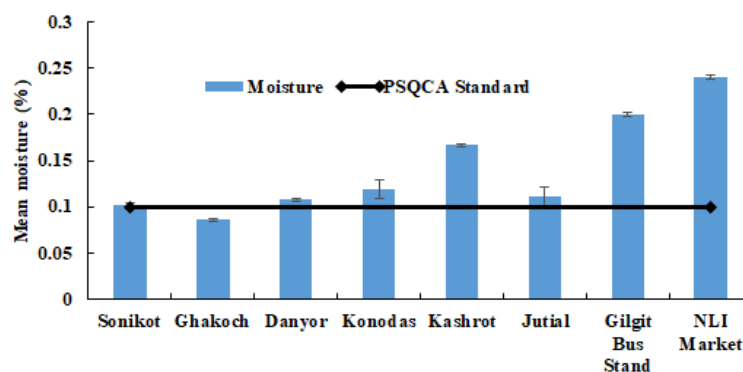


Fig. 2. Mean MC mean values obtained from oil samples from different SFVs.

might be poor packaging, storage conditions, and extensive exposure to light and high temperatures which commonly exist in the Pakistani climate. The present study is in agreement with the findings of Sanli *et al.* [23] that the initial high MC induces more enzymatic hydrolysis in oils due to more activity of the lipase.

3.2.3 FFA of fried oil samples

FFA values obtained from fried oil samples ranged from 0.26 ± 0.16 to 0.52 ± 0.21 % and are shown in (Figure 3). FFA analysis showed that all values

significantly deviate ($p = 0.00$) from the standard values of PSQCA (≤ 0.02 %). The highest trend was observed in Ghakuch at 0.52 ± 0.21 % followed by NLI Market at 0.46 ± 0.20 %, Kashrot at 0.35 ± 0.23 %, Gilgit Bus Stand at 0.33 ± 0.15 %, Danyor at 0.31 ± 0.15 %, Jutial 0.28 ± 0.16 %, Konodas 0.28 ± 0.13 % and Sonikot 0.26 ± 0.16 %. The presence of increased FFA tends to cause toxicological effects on human health such as obesity and type 2 diabetes mellitus. This study is in close relation to the study presented by Sebastian *et al.* [33] reported that about 30–35 % of in-use frying oils and 45–55 % of discarded oil samples were not

acceptable, that were collected from restaurants in downtown Toronto, Canada. The possible causes are excessive hydrolysis and oxidative reactions in oil samples due to extensive frying, exposure to light, lipase activity, and high temperature. The poor neutralization process and hydrolysis of triglycerides and deterioration of oil also increase the amount of short-chain free fatty acids [34].

3.2.4 Color of fried oil samples

The color of the cooking oil is due to carotenoids, zeaxanthins, and other coloring pigments. The standard value of colors set by PSQCA is Y:50-R: 5.0 while the mean observed values in fried oil samples were Y: 54.2, R: 6.05. Color values for oil samples deviated significantly ($p = 0.00$) from standard values (Figures 4a and 4b). The redness of cooking oils is mainly due to the formation of polymers and the yellow color is due to combined peroxide and aldehydes in cooking oils. Another factor is the lipase enzyme, which promotes hydrolytic rancidity with the production of FFA and free radicals which significantly affects taste, color, and aroma by breaking the ester bond [34].

3.2.5 IV of fried oil samples

IV is the relative degree of unsaturation of fatty acids that are part of triacylglycerol in oil components and are used for the classification of oils [35]. The recommended IV is (not less than 80 g/100 g of iodine) for vegetable oil blends according to PSQCA and the value decreased with overheating. The average comparison of IV between different areas of Gilgit, Pakistan (Figure 5) concluded that the NLI market with a maximum value of 87.3 ± 3.7 followed by Danyor 76.2 ± 3.8 , Sonikot 75.7 ± 3.3 , Jutial 68.6 ± 3.4 , Gilgit bus stand 59.7 ± 5.1 and Kashrot 59.2 ± 4.9 . Results showed that IV of all areas did not fall under the limits of PSQCA and only NLI is near the limits. This was because of the multiple deep-frying practices performed that led to a higher unsaturation, and the presence of a higher amount of trans fatty acids in the oil. A study also reported the IV for olive oil, palm oil, corn oil, soya bean oil, sunflower oil, and peanut oil in the range of 75-90, 50-55, 103-135, 120-143, 110-143 and 84-105, respectively [36]. The study also concluded that the decrease in the IV indicates oxidative stress and physicochemical changes during frying which is considered an important quality indicator.

3.2.6 Peroxide value of fried oil samples

PV is considered the measure of active oxygen species bounded by cooking oils [34]. PV helps to estimate the amount of oxidation as it reacts with other molecules resulting in aldehydes and ketones responsible for the development of off-flavor. According to PSQCA, the recommended level of POV is 10 meq/kg (PSQCA, PS:2858-2003). The mean PV of collected oil samples from different areas of Gilgit is presented in (Figure 6). The results showed a significant difference ($p = 0.00$) in the PV of all oil samples. The high trend of PV was observed at Sonikot at 16 ± 5.3 followed by NLI Market at 15.2 ± 2.3 , Jutial at 12.6 ± 7.4 , Danyor at 11.7 ± 5.2 , Kashrot at 10.5 ± 7.6 and Gilgit bus stand 7.5 ± 6.5 . Potential reasons for higher PV could be poor packaging, improper storage conditions, and cooking oils rich in unsaturated fatty acids especially linoleic and linolenic acid. Phenolic compounds and antioxidants tend to suppress the oxidation of cooking oils but their activity is lost due to extensive heating. A study also reported that the PV of corn oil increased to 11.9 meq/kg after 3rd frying, where the PV of 27 out of 35 samples was above the acceptable level [19]. The results of the current study also supported the findings of Mudawi *et al.* [37] in which an increase in PV was reported due to the continuous deep-frying of oil [37-38]. Another similar study conducted in Kashmir, India reported the higher degradation of the 16 fried oil samples with a high PV than the standard value (>10 meq/kg) [39].

3.3 Comparative Analysis of Frying Oils Quality

In this study, a z-test was carried out along with descriptive statistics and correlation analysis while studying all the parameters. z-test was considered an appropriate statistical technique. In this study, 78 samples were statistically analyzed in triplicates and the mean value, standard deviation, z-value, and p-value were at a significant level. The comparison of results from the physicochemical analysis is presented in (Table 2). The results of the survey- analysis predict the negative impact of consuming food products fried in degraded oils and SFVs are not aware of the health effects. The results of the TPM showed an extremely significant difference between mean values ($p = 0.00$) and 94 % of oil samples were found beyond the PSQCA limits. In agreement with this study, another study

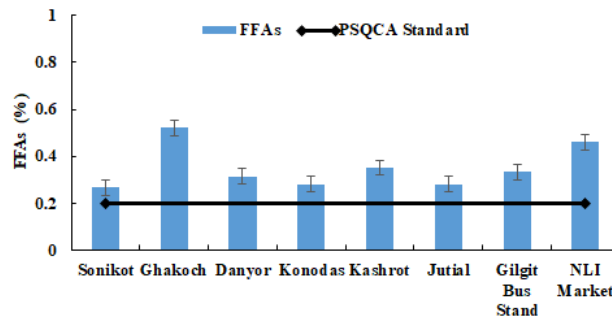


Fig. 3. Mean FFA obtained from oil samples of different SFVs.

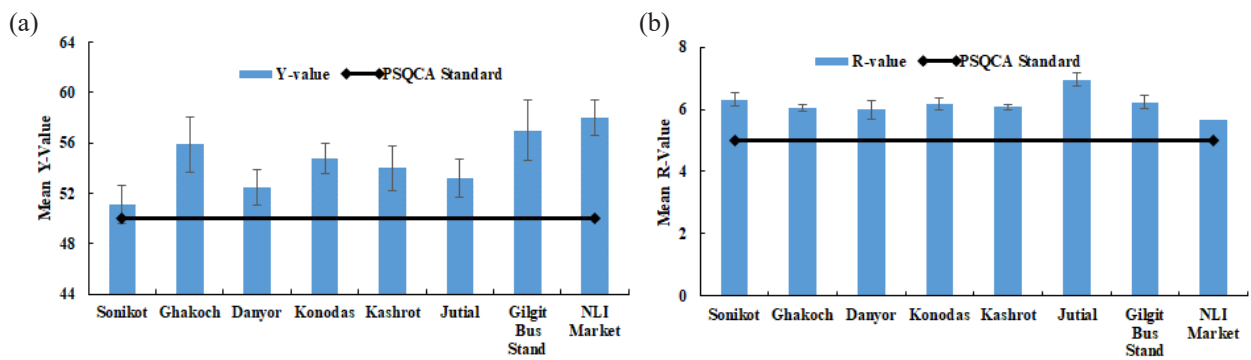


Fig. 4. Colour values of different oil samples (a) R values and (b) Y values.

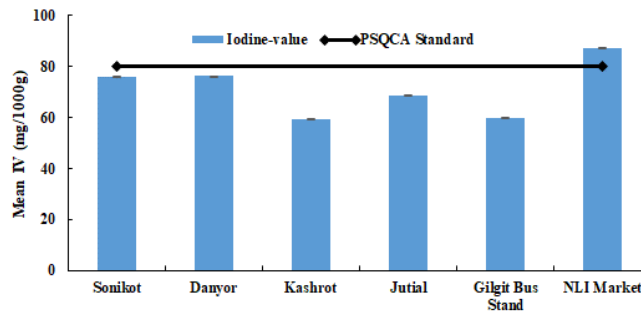


Fig. 5. Mean IV obtained from oil samples of different SFVs.

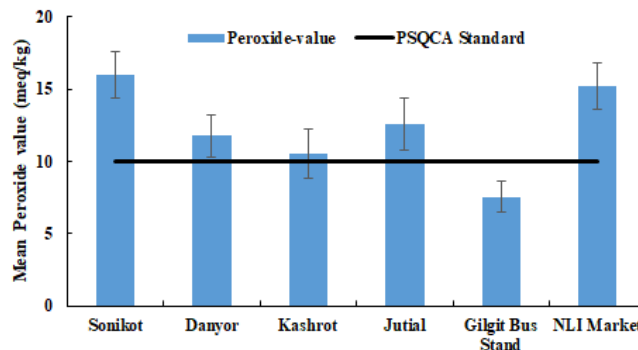


Fig. 6. Mean PV obtained from oil samples of different SFVs.

Table 2. Statistical analysis and comparison of all physicochemical parameters used for qualitative testing of fried oil samples.

Parameters	Test value	Mean	SD	z value	p value	Results
TPM	17.5	19.5	4.28	4.21	0.00	Significant
Moisture	0.15	0.11	0.09	0.65	0.51	Insignificant
FFA	0.20	0.35	0.19	6.72	0.00	Significant
R-value	5.00	6.05	1.48	6.25	0.00	Significant
Y-value	50.0	54.2	5.37	7.04	0.00	Significant
IV	80.0	87.3	2.54	14.4	0.00	Significant
PV	10.0	14.5	2.71	8.30	0.00	Significant

by Kaimal *et al.* [40] reported that there was a significant increase in TPM values found in frying soil samples collected from restaurants in India. It is predominantly highlighted here that TPM exhibit a strong correlation with the number of frying cycles and frying time, this is also in line with studies discussed in previous literature [31-41]. Further, the rate of TPM can be lowered in palm oils due to less unsaturation as compared to soya bean and sunflower oil [31-41]. The results of MC present insignificant relation with frying ($p = 0.051$) and concluded that MC is least affected by frying [31]. On the contrary, results for FFA showed a significant relationship between the high temperature during frying and extended time for all oil samples ($p = 0.00$).

These results are in strongest agreement with the previous study that reported, an increase in frying temperature tends to increase thermal oxidation and oligomerization reactions for fatty acids in oils [42]. Another study also showed that during the thermo-oxidation of oil at frying temperature (> 180 °C), the amount of polyunsaturated fatty acids (PUFA) decreased (reached 6.21 %) with the increase in short chain fatty acids (SCFA) and trans fatty acids with the increase in frying time [43]. Furthermore, the change in R-value and Y-value also validates the significant relation of change in the color of oils at high frying temperatures ($p = 0.00$). Moreover, PV and IV also showed a significant relation with frying oil samples ($p = 0.00$). A similar study in past also reported that the increase in frying time of lipids particularly oils significantly affects the PV in fried clams with concurrent reduction of docosahexaenoic acids and eicosapentaenoic acids that indicate extensive oxidative degradation [44].

4. CONCLUSION

The quality of oils used for frying and fried food items is intimately bounded. The extensive thermo-oxidation and high-temperature cooking degraded the quality of oils. The present study was conducted to develop the combined effect of practices of SFVs while frying and the chemistry of frying oil. For this purpose, the quality of fried oil was evaluated through 78 random samples collected from various SFVs and restaurants in different areas of Gilgit. The results showed that SFVs preferred to use low-quality oils because of low prices and easy availability by compromising the quality. The frying oil was changed infrequently and the used oil was combined with new oil rather than discarded, which can jeopardize the customer's health. The z-test scores of all parameters present that the quality of 78 % of fried oil samples varies from the standard values set by PSQCA, Pakistan. The majority of the frying oil samples exhibited exceedingly high levels of degradation based on TPM, FFA, color, IVs, and PV values. Based on the results, we found that the oil used for frying purposes among SFVs in Gilgit, Pakistan is mainly rancid and this is due to their lack of knowledge about hygienic and sanitary practices. It is recommended that a high-level awareness is required among SFVs and customers on hygienic practices and associated health concerns. To implement suitable frying processes in fast food restaurants, food operators must be trained. It would also be preferable if policymakers designed education initiatives in combination with increased awareness from regulatory bodies that could aid in the improvement in Gilgit, Pakistan. Regulatory bodies must ensure quality control on SFV practices and should often monitor for the sake of public health interests. It is also suggested

that prior to receiving a restaurant license, sufficient training and awareness sessions must be conducted. The current research can be extended further by evaluating the quality of and nutritional evaluation of the oil absorbed by fried food items because the present situation is found quite alarming.

5. CONFLICT OF INTEREST

The authors declared no conflict of interest in writing this article.

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Growth Analysis of Production of Food Crops and Population Growth for Food Security in Pakistan

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Abstract: Food availability is one of the important pillars of food security. It is essential to ensure food availability in the world to avoid starvation. The key concern of the present study is to integrate the fluctuations in the growth rate of the population and important food crop production using time series data from 1950-2021. Semi-log compound growth rate models are applied for the projection, Cuddy-Della and Valle instability index are used for the stability analysis, and decomposition analysis models are applied to determine the contribution of factors toward production. Semi log compound growth rate model indicates that the population of Pakistan is increasing rapidly, while the contribution of productivity and area of all food crops are not enough to meet food sustainability. The growth rate in the area and production of Wheat, Rice, and Maize are positive, while it is negative for Sorghum (jawar), Millet (bajra), and Barley. For areas, a low degree of instability is prevailing for Wheat, Rice, Maize, Sorghum (jawar), and population, while the medium is for Millet (bajra) and Barley. For yield, the degree of instability is low for all food crops except Maize, which lies in the medium instability index. Semi log compound growth rate model was found best fitted for area and productivity for all food crops, while for the production side, it is found best for Wheat, Rice, and Maize and bit fit for Sorghum (jawar), Millet (bajra) and Barley. Decomposition analysis model predicted that crop productivity is a major concern to attain food security in Pakistan.

Keywords: Food Crops, Food Security, Growth Rate, Instability, Non-Linear Model, Population.

1. INTRODUCTION

Agriculture is the biggest pillar of Pakistan's economy. World Food Summit, 1996 defines food security, as "when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life" [1]. FAO further characterized food security into four important pillars, "food availability, food access, food utilization, and food stability" [2-4]. These pillars of food security are interrelated with agricultural production [5]. Food availability is however a basic pillar of food security, which depends on the production of food crops [6]. In the context of food availability, Wheat, Rice, and Maize are considered staple food crops. Agriculture sector was contributing 53 % to Pakistan's GDP

in 1949, but over time the share of agriculture in Pakistan's economy has reduced to 19.2 %, which has also reduced the share of the labor force engaged in agriculture from 65 % to 38.5 %. The growth rate of the population in Pakistan is high as compared to neighboring South Asian countries [7]. The high growth rate of the population is leading Pakistan towards a food shortage and may affect the adequate food availability for the growing population [7, 8]. According to UNO, MDGs, food security has become a global threat to the world, due to the expansion of the world population. Food requirements and population growth are directly interlinked with each other [9, 10]. In 2005-06, 16 % of the world population was hungry, and now it has become 21 %, especially in South Asian countries [11]. It is expected that in 2050, Pakistan will be 5th populous state in the world instead of

6th, due to the high growth rate of the population [12]. In 1798, Malthus postulated the theory that the population of the world is increasing rapidly while agriculture production is not enough to meet the need for food for the world, and the world will have to face the severe food scarcity in near future [13, 14]. International organizations i.e. WHO, and FAO show their great concern for this terrible issue of food security [15]. The high growth of the population has become a major threat to food security which can be overcome by increasing agricultural output [16]. The world's population will reach 9 billion by 2050, and the major contribution to this increase will arise from developing countries [15, 17]. The world agriculture production of food should be increased by about 70 % to attain food security and for developing countries, it will double [18]. Murindahabi [19] applied compound growth rate, decomposition analysis, and instability measures, and concluded that there exists a positive and significant relationship between area and production of Rice, Wheat, Beans, Millet, Maize, Groundnut, and Peas. The area contributed majorly toward crop production. Agriculture sector has great potential to grow more food for the population. There should be enforced agriculture emergencies in developing countries like Pakistan. A comprehensive program is needed to increase agriculture productivity, and to make food security measures.

It is the foremost need of the time, is to statistically measure the growth rate of the population and food crops to make assure food availability, and to elaborate the future food concerns. The present study statistically compared the population growth rate of six food crops (Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra), and Barley using the time series data comprise from 1950-2021, to address the food security situations in Pakistan. This study uses an exponential regression model (compound growth rate model), instability measure index (CDVI), and decomposition analysis model for area, productivity, and production of food crops (Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra), Barley along with the comparison of population, and statistically forecast the area, yield and production of food crops and population of Pakistan. This study will lead us to understand the true policy decisions to combat the food security measures in Pakistan.

2. MATERIALS AND METHODS

2.1 Data Collection and Measuring Scales

The seventy-two-year secondary time series data comprise from 1950-2021 is gathered from the Punjab agriculture marketing information service department, Pakistan bureau of statistics and crop reporting service agriculture department Punjab for the food crops (Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra) and Barley) of Pakistan. These are government organizations responsible for the collection and publication of statistical data for research and policymakers. The data measuring scale is applied as area sown in thousand acres, production in thousand tonnes and yield in mounds per acre (mds/acre).

2.2 Non-Linear Compound Growth Rate Regression Model (CGRM)

The non-linear compound annual growth rates regression model (CGRM) is used to elaborate the changes in food crops area, productivity, and production along with comparisons of population, and it is measured by log-linear function [20-22]

$$z_t = z_0 [1 + r]^t \quad (1)$$

Where, “ z_t ” shows food crops area/productivity/production and population, “ t ” represents the time, “ 0 ” is used for the initial time, and “ r ” is used to indicate the growth rate of determinates. The slope measures the relative change in response variable for the absolute change accrues in feature and it measures the instantaneous rate of growth. Apply the natural log on equation 1

$$\ln(z_t) = \ln(z_0) + t\{\ln(1 + r)\} \quad (2)$$

$$\begin{aligned} \ln(z_t) &= Y, \quad \ln(z_0) = A_0, \quad \ln(1 + r) = V, \\ r &= (\exp^V - 1) * 100 \\ Y &= A_0 + Vt + \varepsilon \end{aligned} \quad (3)$$

The following equation is applied to predict the parameter.

$$z_p = [z_c (1 + V)^{tn1 - tn2}] \quad (4)$$

Where, “ z_p ” stands the value of the response

variable at a projected time, “ z_c ” stands the actual value of response at the time “ t ”, “ V ” stands for regression coefficients, and “ $tn1-tn2$ ” stands the total no. of projected years.

2.3 Instability Analysis

Cuddy Della and Valle index (CDVI) is applied to measure the instability in time series data. It attempts to de-trend the coefficient of variation (CV) by R^2 . The low value of CDVI elaborates on the low instability and vice-versa. Instability will be low if CDVI lies in $0 \leq CDVI \leq 15$, medium if CDVI lies in $15.1 \leq CDVI \leq 30$, and high if CDVI lies as $CDVI > 30$ [23].

$$CDVI = C.V \times (\sqrt{1 - R^2}) \quad (5)$$

$$C.V = \frac{s}{\bar{x}} \times 100 \quad (6)$$

Where “ s ” stands for the standard deviation of the data and “ \bar{x} ” stands the mean value of the data.

$$R^2 = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y}_i)^2} \quad (7)$$

$$R^2 = 1 - \frac{SSR}{SST} \quad (8)$$

Where “SSR” stands the sum of squares of regression and “SST” stands the total sum of squares,

$$R^2_{Adjusted} = 1 - \frac{(1 - R^2)(n - 1)}{n - k - 1} \quad (9)$$

Where “ n ” stands the no. of observations and “ k ” stands the no. of predictors in the model.

2.4 Decomposition Analysis Model

The production of the crop is the product functions of the area and yield of the respective crop. Decomposition analysis model is used to estimate the individual share of the area and yield towards the production [19]. The variation in production is the sum of individual share of area, yield, and their interaction effects which is measured as;

Production change = Yield effects + Area effects + Interaction effects

$$\Delta P = A_0 \Delta Y + Y_0 \Delta A + \Delta Y \Delta A \quad (10)$$

$$\text{Change in production} = \frac{(Y_c - Y_0) \times A_0}{P_c - P_0} \times 100 + \frac{(A_c - A_0) \times Y_0}{P_c - P_0} \times 100 + \frac{(Y_c - Y_0) \times (A_c - A_0)}{P_c - P_0} \times 100$$

Where “ $\Delta P = P_c - P_0$ ” = Change accrue in production over a period of time, “ $\Delta Y = Y_c - Y_0$ ” = Change accrue in productivity over the period, “ $\Delta A = A_c - A_0$ ” = Change accrue in the area over time, “ 0 ” = base year, “ c ” = current year.

3. RESULTS AND DISCUSSION

3.1 Non-Linear Compound Growth Rate Regression Model (CGRM)

Food security is closely linked with the production of major food crop growth in Pakistan [24]. Wheat, Rice, and Maize are the staple food crop of Pakistan. Table 1, elaborates the coefficients of determinations, regression coefficients, CGR, predicted values, and regression diagnostics of fitted CGRM for the food crops area and population, while Table 2, shows these determinates for the food crops productivity and production. Wheat, Rice, and Maize crops are accounting 1.8 %, 0.5 %, and 0.7 % share of the GDP of Pakistan [25]. In 2021, Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra), and Barley are cultivated on 22655.66, 8242.56, 3503.51, 310.3, 865.87 and 103.39 thousand acres areas in Pakistan, while it was 10337, 2305, 989.63, 1361, 2368 and 497 thousand acres in 1950. Using the CGRM, the growth rates for area reported as 1.21 %, 1.61 %, 1.71 %, -1.39 %, -1.29 %, and -1.69 %, respectively for Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra) and Barley. The productivity in mds/acre of Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra), and Barley were 9.34, 8.59, 10.13, 4.9, 3.91, and 7.34 in 1950 and it is reported as 30.3, 25.54, 63.79, 7.73, 7.68 and 10.12 in 2021. The models predicted the growth rate for the productivity as 2.22 %, 1.71 %, 2.43 %, 0.50 %, 0.70 %, and 0.80 %, respectively for the Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra) and Barley crops. For the production side, Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra), and Barley reported 3862, 792, 401, 267, 370, and 146 thousand tonnes in 1950, and these crops accounting production as 27464.08, 8419.68, 8939.79, 95.97, 266.08 and 41.86 thousand tonnes for 2021. The fitted model predicted the production

Table 1. Fitted CGRM determinants for food crops area and population of Pakistan

	Area in acres all food crops						Population in million
	Wheat	Rice	Maize	Sorghum (Jawar)	Millet (Bajra)	Barley	
Adj R ²	0.916	0.931	0.965	0.734	0.637	0.679	0.993
MSE	0.006	0.009	0.005	0.029	0.039	0.059	0.002
F-Statistic	779.2**	957.4**	1553.0**	196.7**	125.8**	151.3**	9798.7**
Slope (β)	0.012	0.016	0.017	-0.014	-0.013	-0.017	0.028
t-Statistic	27.9**	30.9**	44.19**	-14.02	-11.2	-12.3	98.9**
CGR	1.21%	1.61%	1.71%	-1.39%	-1.29%	-1.69%	2.84%
1950	10337	2305	989.63	1361	2368	497	32.92
2021	22655.66	8242.56	3503.51	310.3	865.87	103.39	224.78
2030 (Predicted)	25223.27	9508.36	4077.48	273.32	769.67	88.61	288.20
2050 (Predicted)	32019.28	13061.10	5712.30	206.16	592.45	62.88	500.68
% Inc/Dec at 2030	11.33%	15.36	16.38	-11.92	-11.11	-14.30	28.21%
% Inc/Dec at 2050	41.33%	58.46%	63.05%	-33.56%	-31.58%	-39.18%	122.74%

** Shows the models and coefficient are statistically significant

Table 2. Fitted CGRM determinates for food crops productivity and production.

	Productivity (Yield) in mds/ acre					
	Wheat	Rice	Maize	Sorghum (Jawar)	Millet (Bajra)	Barley
Adj R ²	0.949	0.898	0.818	0.644	0.635	0.719
MSE	0.012	0.014	0.054	0.005	0.014	0.012
F-Statistic	1324.7**	629.4**	319.9**	129.2**	124.4**	182.2**
Slope (β)	0.022	0.017	0.024	0.005	0.007	0.008
t-Statistic	36.3**	25.0**	17.8**	11.3**	11.1**	13.4**
CGR	2.22%	1.71%	2.43%	0.50%	0.70%	0.80%
1950	9.34	8.59	10.13	4.9	3.91	7.34
2021	30.31	25.54	63.79	7.73	7.68	10.12
2030 (Predicted)	36.87	29.72	78.97	8.08	8.18	10.87
2050 (Predicted)	56.97	41.64	126.90	8.93	9.40	12.75
% Inc/Dec at 2030	21.63	16.38	23.79	4.59	6.48	7.43
% Inc/Dec at 2050	87.96	63.05	98.93	15.56	22.42	26.00

	Production in tonns of all food crops					
	Wheat	Rice	Maize	Sorghum (Jawar)	Millet (Bajra)	Barley
Adj R ²	0.952	0.934	0.941	0.473	0.134	0.286
MSE	0.026	0.033	0.045	0.038	0.071	0.08
F-Statistic	1396.2**	1013.5**	1142.3**	64.7**	11.9**	29.4**
Slope (β)	0.034	0.033	0.041	-0.009	-0.005	-0.009
t-Statistic	37.3**	31.8**	33.7**	-8.04	-3.4	-5.4
CGR	3.46%	3.36%	4.19%	-0.9	-0.5	-0.9
1950	3862	792	401	267	370	146
2021	27464.08	8419.68	8939.79	95.97	266.08	41.86
2030 (Predicted)	37106.49	11277.13	12834.65	88.47	254.34	38.59
2050 Predicted)	72420.36	21587.63	28668.07	73.84	230.08	32.21

** Shows the models and coefficient are statistically significant

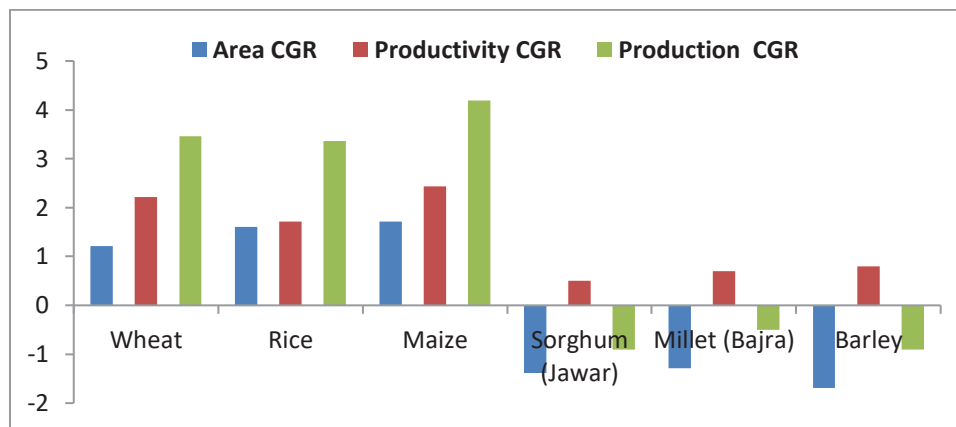


Fig. 1. Graph showing CGR for food crops

growth rate as 3.46 % for Wheat, 3.36 % for Rice, 4.19 % for Maize, -0.90 for Sorghum (jawar), -0.50 for Millet (bajra), -0.90 for and Barley. Figure 1, shows the graphical view of the growth rates of all the food crops. The population of Pakistan is reported 224.78 (million) in 2021, while it was 32.92 (million) in 1950. The CGRM reported a growth of 2.84 % for the population.

The fitted CGRM shows explained variation as 91.6 %, 93.1 %, 96.5 %, 73.4 %, 63.7 %, 67.9 %, and 99.3 % respectively for foods crops (Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra) and Barley) areas and population of Pakistan. Fitted models show explained variations as 94.9 %, 89.8 %, 81.8 %, 64.4 %, 63.5 %, and 71.9 % for productivity, and 95.2 %, 93.4 %, 94.1 %, 47.3 %, 13.4 % and 28.6 % for productions of Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra) and Barley. F-statistic shows all fitted CGRM is significant. The t-statistic shows regression coefficients are significant for all food crop productivity. The regression coefficients were found significant for the area and production of Wheat Rice and Maize crops and insignificant for the Sorghum (jawar), Millet (bajra), and Barley. Fitted population regression coefficients were found significant. The slope and CGR for area and production were found negative for Sorghum (jawar), Millet (bajra), and Barley, and positive for Wheat, Rice, and Maize crops. The slope and CGR were found positive for the productivity of all food crops. The population will reach 288.20 (million) by 2030 and 500.68 (million) by 2050. CGRM was found best fitted for the area and productivity of all food crops. For the production side, CGRM, the best fit for Wheat, Rice, and Maize and bit fit for

Sorghum (jawar), Millet (bajra), and Barley crops. Keeping in view of major food crops (Wheat, Rice, and Maize crops) and the population of 2050, the increase in population is 81.41 %, 64.28 %, and 59.69 % greater than from the Wheat, Rice, and Maize area. The area under Sorghum (jawar), Millet (bajra), and Barley were reported to decrease by 33.56 %, 31.58 %, and 39.18 %, while the population is increasing by up to 122.74 % by 2050. The increase in population reported 34.78 %, 59.69 %, 23.81 %, 107.18 %, 100.32 %, and 96.74 % greater than from Wheat, Rice, Maize, Sorghum (jawar), Millet (bajra) and Barley crops productivity up to 2050. The increase in population is greater than the increase in area and productivity of all food crops which shows the expected food stress for Pakistan.

3.2 Growth Rate and Instability Measures of Food Crops

Table 3 shows the instability analysis using the Cuddy Della and Valle index (CDVI) and coefficient of variations for food crops and the population of Pakistan. Regarding the area of food crops, low instability was found for Wheat, Rice, Maize, and Sorghum, while medium for Millet and Barley. The instability was found lower in all food crop productivity except for Maize, which is medium. For production side instability, all food crops lie in the medium instability index except Wheat, which lies in the lower instability index. The coefficient of variation indicates high inconsistency for population compared with area and productivity of food crops.

3.3 Decomposition Analysis Model for Area and Productivity towards Production

Production is the sum of the product of area and productivity (area*productivity). Decomposition model is used to determine the contribution of area and productivity toward production. It is shown in Table 4, Figure 2, that productivity is the main contributor to change in all food crop production as compared to the area. For Wheat, Rice, and Maize, productivity effects were found 18.24 %, 6.28 %, and 12.96 % greater than from area. The contribution of the area is low as compared to

productivity (Productivity effects > Area effects) and in some cases, the crop area is critically negative for Sorghum (jawar), Millet (bajra), and Barley. The area and productivity both collectively contribute towards production and accounting 43.76 % share of Wheat, 52.76 % of Rice, and 63.20 % of Maize. The interaction effects were found negative for Sorghum (jawar), Millet (bajra), and Barley crops. Productivity contributes an upswing for all food crops. Interaction effects for Sorghum (jawar), Millet (bajra), and Barley were found negative due to the negative growth rate for the area of these crops.

Table 3. CDVI for the food crops area, productivity, and production

	Area		Productivity		Production		Population	
	CDVI	CV	CDVI	CV	CDVI	CV	-	-
Wheat	6.80 %	23.5 %	9.8 %	43.1 %	13.50 %	60.0 %	-	-
Rice	8.50 %	32.6 %	10.6 %	32.3 %	15.3 %	59.5 %	-	-
Maize	6.43 %	34.4 %	29.18 %	60.0 %	25.50 %	105.2 %	-	-
Sorghum (Jawar)	14.76 %	28.6 %	6.8 %	10.9 %	17.50 %	24.2 %	-	-
Millet (Bajra)	19.69 %	32.6 %	12.3 %	16.8 %	24.30 %	26.1 %	-	-
Barley	19.70 %	34.8 %	10.4 %	19.6 %	25.10 %	29.8 %	-	-
CDVI	-	-	-	-	-	-	4.44 %	
CV	-	-	-	-	-	-	53.3 %	

Table 4. Decomposition analysis for area and productivity share toward production

	Area effects	Productivity effects	Interaction effects
Wheat	19.48 %	37.72 %	43.76 %
Rice	20.48 %	26.76 %	52.76 %
Maize	11.92 %	24.88 %	63.20 %
Sorghum (Jawar)	-43.00 %	32.17 %	-24.84 %
Millet (Bajra)	-28.69 %	43.60 %	-27.65 %
Barley	-53.86 %	25.76 %	-20.40 %

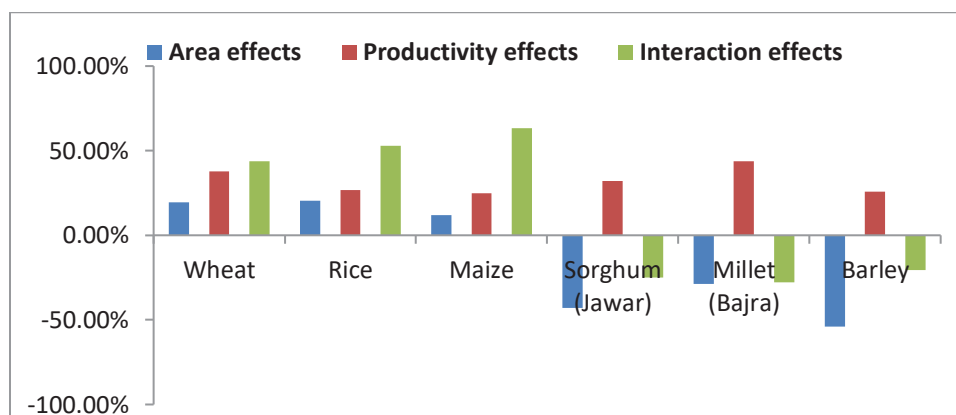


Fig. 2. Graph showing the decomposition analysis model for food crops

4. CONCLUSION & RECOMMENDATIONS

Food availability is one of the important pillars of food security. It is essential to steer the strategies to ensure food availability. The population of Pakistan is increasing rapidly, while the productivity and area of all food crops are not enough to meet food sustainability. The key concern of the present study is to statistically analyze the growth rate of the population and important food crop production fluctuations using time series data comprise from 1950-2021. The growth rate in area and production of Wheat, Rice, and Maize are positive, while negative for Sorghum (jawar), Millet (bajra), and Barley. The growth rate is positive for all food crops' productivity. The growth rate in area and productivity of all food crops is less than the growth rate of the population. The degree of instability is low for the area of Wheat, Rice, Maize, and Sorghum (jawar), while medium for Millet (bajra) and Barley. Low degree of instability prevails for the productivity of all food crops except Maize, which is medium. The production side instability is medium for all food crops except Wheat, which is low. Decomposition analysis model indicates productivity is a major concern to increase the production of crops to meet the challenge of food security in Pakistan. CGRM is best fitted for the area and productivity of all food crops. The population will increase by 81.41 %, 64.28 %, and 59.69 % greater than from Wheat, Rice, and Maize areas up to 2050. The area under Sorghum (jawar), Millet (bajra), and Barley are reported to decrease, while the population is increasing by about 122.74 % by 2050. The increase in population is greater than the increase in area and productivity of all food crops which shows the expected food stress for Pakistan. The comparison of instability, growth and decomposition analysis for food crops and population will lead the government to lay out direction-oriented policies to attain food sustainability for future food security concerns. The comparison of decomposition analysis will lead the government to that productivity enhancement is a major concern to ensure food availability in Pakistan. The comparison of area and productivity will lead the government to rationalize the policies by keeping in view the expected increase and decrease across the years. This study could also enhance to compare the periodic comparisons and some other linear and nonlinear models could also be applied to compare the results of fitted models.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declared no conflict of interest

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A Cross-Sectional Study Elucidating Associated Predictors in Postpartum Depression among Pakistani Women

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Abstract: Postpartum depression is a psychological condition that deteriorates a mother's cognitive health and overall family functioning. This survey-based cross-sectional study was done on postpartum mothers between 15-40 years of age to determine the epidemiology of postpartum depression in Pakistan. Edinburg postnatal depression scale (EPDS) and an adapted relationship assessment tool were used to determine the prevalence of postpartum depression. A total of n=103 responses were collected from different regions of Pakistan. Univariate and Bivariate analyses, Pearson Correlation Tests, and Binary Logistic Regression Analyses were applied to investigate the predictors. Postpartum depression was found in 67.96 % of the participating women and EPDS was found to be the most reliable tool to evaluate postpartum depression.

Keywords: PPD, EPDS, Gestational, Women's health, Depression, Stress, Postpartum, Disorder

1. INTRODUCTION

Women undergo different reproductive stages and hormonal changes throughout their life. Childbirth and the postnatal period are major transformations, in which women face various complications including heavy vaginal bleeding, vaginal discharge, fever, lower abdominal pain, perineum, and extreme fragility.

During this phase, various hemodynamic, genitourinary, metabolic, and emotional changes occur in the female's body [1]. These variations eventually lead to various psychological changes which may cause debilitating mental disorders such as postpartum depression. Postpartum depression (PPD) is a non-psychotic depressive mood disorder that deteriorates a mother's mental health and ultimately affects her family life by impairing her ability to endure various life challenges including parenting [2]. Post-partum depression (PPD) could be manifested by various physical and emotional symptoms initiated within 2-4 weeks of childbirth and may have severe outcomes for both mother and infant including maternal mortality and morbidity,

increased risk of infanticide, and poor attachment between the mother and her child [3]. Different diagnostic systems may define postpartum depression in different ways as the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) categorizes it as a major depressive disorder whose symptoms may begin within four weeks postpartum while the International Statistical Classification of Diseases and Related Health Problems (ICD - 10) defines it as a mild mental and behavioral disorder having an onset within six weeks postpartum [1]. The symptoms may include severe mood swings, irritability, insomnia, lethargy, anxiety, difficulty bonding with the baby, feeling of worthlessness, appetite problems, recurring anxious and suicidal thoughts, reduced concentration, short-term memory loss, paranoia, thoughts of killing the baby, hallucinations, disorientation, self-isolation, and hyperactivity. Women who are at risk of developing depressive conditions are rarely identified during pregnancy or at the time of delivery [4-6].

The World Health Organization states that about 10 % of pregnant women around the world

and 13 % of the women who recently gave birth experience some type of mental disorder. The prevalence of PPD across Asian countries varies (3.5-63.3 %); Malaysia has the lowest rates (<4 %), while Pakistan has a wide range of extremely higher rates ranging from 28 % to 94 % in different studies [7-13]. Higher rates of PPD could be due to conflicting recommendations from family, strict cultural norms, illiteracy, and poor health facilities. There may sometimes be quite different opinions and demands from the family regarding the care of the baby and mother. Often it causes considerable stress when mothers follow the traditional structures while at the same time health care professionals with contradictory beliefs impose their advice that seldom seems to be reasonable for the mothers [14].

Generally, the important factors involved in the prevalence of PPD could be divided into various categories: Biological/ physical factors, psychological factors, pediatric factors, socio-demographic factors, and cultural factors [15, 16]. These include prenatal anxiety, spouse relationship, family support, income problems, health issues, gestational age, and various other living conditions. In addition, a history of previous episodes of PPD, marital conflict, and single parenthood are also predictive of PPD [17, 18].

In the onset of postpartum depression, multiple factors including genetics, epigenetic modifications, neuroactive molecules, and environment play their role [19]. The heritability of PPD is approximately 50 % which indicates a significant genetic contribution suggesting genetic etiologies [20]. Similarly, different DNA methylation biomarkers have been identified which play important role in the onset of postpartum depression as the epigenetic modifications due to stress, medications, and reproductive hormones may cause DNA methylation which makes it of particular interest to discover the onset of postpartum depression [21]. Studies have indicated that various neuroactive molecules such as reproductive hormones including allopregnanolone and brexanolone, γ -aminobutyric acid (GABA), brain-derived neurotrophic factors (BDNF), and beta-endorphins may play important role in the onset of postpartum depression [19, 22, 23]. Adverse life events having a significant impact on women's physical and mental health and psychiatric history are the most validated and

repeated predictors discussed in the literature. Moreover, eating habits, proper nutrition intake, and various demographic factors including age and a number of children may play an important role in the onset of postpartum depression [19].

The majority of the patients seeking mental healthcare are women (69 %) that may get treatment for neurotic, stress-related, and mood disorders. Yet, mental health facilities receive only 0.4 % of the share of the total healthcare budget. However, the diagnosis and treatment of postpartum depression are not included in these healthcare facilities. Thus, it remains under-diagnosed [1].

So far, not many studies have been conducted in Pakistan that represents the overall prevalence of PPD at provincial and national levels. The cultural norms do not pay serious attention to these psychosocial women's health issues along with the lack of reliable screening tools for PPD; therefore, the cases remain undiagnosed and untreated [10]. Not many women get diagnosed with the development of PPD based on the symptoms and thus do not receive proper treatment for the prevailing condition.

For the diagnosis of postpartum depression, certain scales and screening tools have been developed. Edinburgh postnatal depression scale (EPDS) is the most widely used and reliable tool to investigate the prevalence of postpartum depression having a 92 % response rate, 79 % sensitivity, and 85 % specificity [24]. Moreover, other scales including WHO Schedule for Clinical Assessment in Neuropsychiatry (SCAN), Personal Information Questionnaire (PIQ), and Brief Disability Questionnaire (BDQ) have been used by various researchers [1, 7, 8, 10]. Moreover, the relationship of the women with the spouse can be assessed by the relationship assessment tool and women experiencing with battering scale (WEB) to determine the quality of life and effect of behaviors in the development of postpartum depression.

The knowledge of prevalence rates and important indicators of PPD are essential for the implementation of preventative measures and to address the at-risk groups. Herein, we evaluated characteristic predictors for PPD among Pakistani women ranging between the ages of 15-40 years.

2. MATERIALS AND METHODS

2.1 Study Design

A cross-sectional study design was used to evaluate the factors playing a significant role in the prevalence of post-partum depression. A closed-ended questionnaire was developed, both in English and Urdu language as a data collection tool so that information about the challenges faced during the postpartum period could be collected from the mothers currently in their postnatal period. The questionnaire had 4 sections. First was about the general information; second included their family life experiences post-partum; third section consisted of an adapted relationship assessment tool (Figure 1). The tool consisted of 10 questions and was formulated by merging questions from three different scales including (1) Women's Experiencing Battering (WEB) [25], (2) Hit-Insert-Threaten-Scream (HITS) [26], and (3) Relationship Assessment Tool (RAT) [27-29]. The fourth section evaluated depression by Edinburgh Postnatal Depression Scale (EPDS) [30]. Scoring was done based on the Likert scale (Tables 1 & 2). The questionnaire was circulated via various online platforms and the data was collected.

2.1.1 Inclusion Criteria

The participants were mothers between 15-40 years of age who recently gave birth. The study participants were from different provinces of Pakistan i.e., Punjab, and Khyber Pakhtunkhwa (KP). However, no one participated from the province of Baluchistan and Sindh.

2.1.2 Exclusion Criteria

The exclusion criteria were as follows: (1) Unmarried women who have no experience of parturition, (2) Married women but haven't experienced a parturition period, (3) Women experiencing their first pregnancy but haven't given birth yet, and (4) Women who faced miscarriages and does not have any children. (5) Mothers having children with genetic disorders. Patients who met the inclusion and exclusion criteria were asked to fill in the designed questionnaire.

2.2 Data Management

A total of n=103 responses were collected and the data after the quality examination was entered using Microsoft Excel. The analysis of the data gathered was completed using the Statistical Package for the Social Sciences (SPSS), Version 21 (IBM, Chicago, Illinois, USA). Univariate and bivariate analysis was performed for all the variables to examine the association of postpartum depression to various other factors. To analyze the main predictors of postpartum depression, a multivariate analysis was performed using binary logistic regression in which model variables were included that showed significant associations between the dependent and independent variables in the bivariate analysis with the confidence limit kept at 5 %.

2.3 Outcome and Predictor Variables

To examine the prevalence of PPD, the status of the mother with respect to PPD was taken as the dependent variable. Various other physical, psychological, socio-demographic, and pediatric factors which could influence postpartum depression significantly as recruited by the scales used literature review were taken as independent variables. The fundamental maternity-related data included the gestational age of mother, no. of pregnancies, history of miscarriages, and the number of children and breastfeeding status of mothers.

Univariate and Bivariate analyses, Pearson Correlation Tests, and Binary Logistic Regression Analyses (Figure 2) were applied to investigate the predictors.

3. RESULTS

We received 103 responses from women ages between 15-40 years out of which 67.96 % were positive for post-partum depression. The sensitivity of our questionnaire incorporating an adapted relationship assessment tool and Edinburgh postnatal depression scale along with the socio-demographic information came out to be 88.4 %, specificity 73.5 %, and accuracy 83.4 %. The odds ratio calculated OR=21.18 indicated that the odds of development of postpartum depression in the

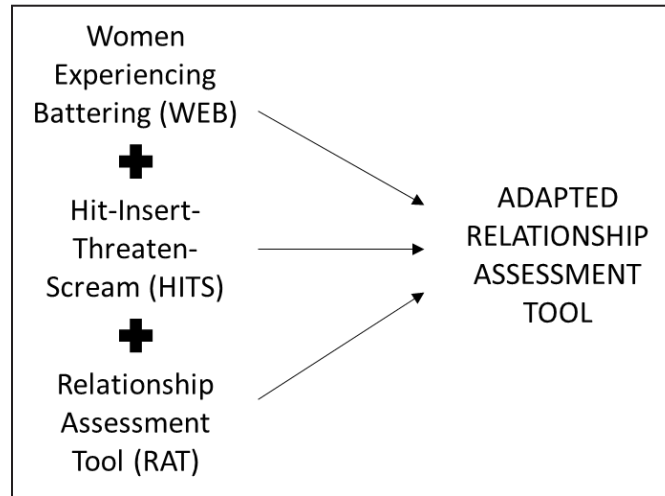


Fig. 1. Adapted relationship assessment tool

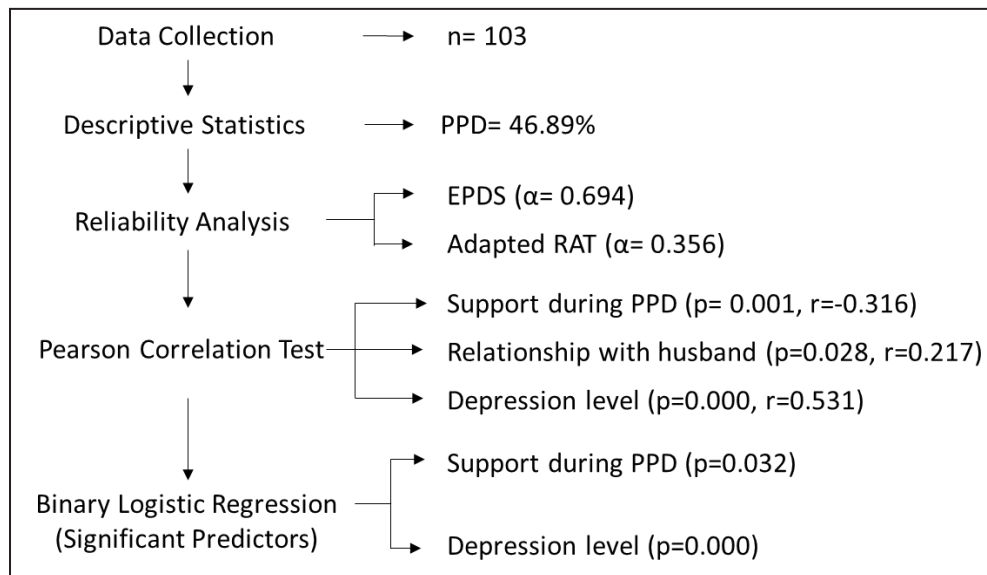


Fig. 2. Schematic description of methodology and results








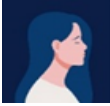


women self-claiming about having depression were 21.18 times higher as compared to those women feeling content with their physical, psychological, and socio-demographic factors. The SD score of postpartum depression came out to be 46.89 %. The majority of the women who were predicted to be positive for PPD had ages ranging between 30-40 years and the mean marrying age of the women who responded was 24.1942 (SD=3.4699). Most of the women claimed to have experienced postpartum depression with the birth of their first child. Eighty percent (80 %) were university level literate, 64.28 % were non-working women; 65.97 % of the women experiencing post-partum depression lived in urban areas while 100% women of the rural

areas were predicted to be positive for postpartum depression. About 47 % of the women in the joint family system experienced PPD whereas 53 % of the women having nuclear family systems had PPD. Observations ascertain that family history, history of miscarriages, abusive relationships, and incorporation of physical activities in daily life played essential roles in the aggravation of post-partum depression. Most of the mothers who were predicted positive for PPD and claimed to have various health issues playing a role in the triggering of PPD ranged between 36-40 years of age. Moreover, the age gap between the children of these women ranged between 6-15 years.

Table 1. Assessment of Relationship of Women by the adapted relationship assessment tool

Type of Relationship	Never (%)	Sometimes (%)	Frequently (%)
 Argumentative	9	66	25
 Insulting	50	36	14
 Hurtful	95	4	1
 Threatening	78	17	5
 Spoken Violence	60	31	9
 Physically Abusive	36	41	23
 Commanding	85	12	3
 Strangled	80	12	9
 Empowering	2	12	81
 Lacking Communication	6	17	74

Table 2. Assessment of Postpartum depression by Edinburgh postnatal depression scale (EPDS)

	Depression Traits	Never (%)	Sometimes (%)	Frequently (%)
	Happiness	14	66	20
	Social Interests	5	71	24
	Self-Accusation	34	58	8
	Stress	38	50	12
	Fear	30	52	18
	Over-thinking	55	40	5
	Insomnia	28	54	18
	Sadness	36	47	17
	Crying	32	47	21
	Self-Harm	9	31	60

The reliability of the Edinburgh Postnatal Depression Scale and adapted Relationship Assessment Tool in determining post-partum depression was indicated by Cronbach's alpha values of 0.694 and 0.356 respectively. This indicated that Edinburgh Postnatal Depression Scale was a reliable source of predicting postpartum depression whereas the adapted Relationship Assessment Tool was not a very reliable source to

analyze PPD.

P-values of the Pearson Correlation test demonstrated that support during the post-partum depression ($p=0.001$), relationship with the husband ($p=0.028$)—was assessed by the adapted Relationship Assessment Tool with a cutoff value of 11. The higher values indicated bad marital relationship status of the women-- and depression

level ($p=0.000$) assessed by Edinburg Postnatal Depression Scale with a cutoff value of 13, indicating depression in those women having score greater than 13 whereas those having depression score (DS) values above 10.5 also had the symptoms of mild depression--were significant contributors of postpartum depression. Moreover, a correlation value of 0.531 depicted a positive correlation between depression score and post-partum depression which meant the higher the depression score by EPDS, the higher the Postpartum Depression. Correlation value of 0.217 for relationship status indicated that the higher the relationship assessment scores depicting a bad relationship, the higher the postpartum depression. Whereas the correlation value of post-partum support (-0.316) manifested that a negative correlation existed between this variable and post-partum depression. This indicated that the higher the level of postpartum support attained by the mother, the lower the development of postpartum depression.

Regression Analysis

The Correlation analysis showed a significant correlation of three factors (depression score, postpartum support score, and relationship assessment score) with postpartum depression. Therefore, Binary Logistic Regression Analysis was performed and the Hosmer-Lemeshow test for goodness of fit was used along with exp (B) with 95% CI to determine the number of times PPD could be affected by various significantly contributing predictors. Hosmer Lemeshow’s test for goodness of fit having a p-value of 0.885 indicated that the selected model was a good fit. The analysis predicted that two factors (depression score and postpartum support score) out of those previously determined three correlated factors were the significant predictors of postpartum depression. The probability of acquiring post-partum depression

by taking depression score, relationship assessment score, and post-partum support into account came out to be 42.5 %. Depression score (p value= 0.000 , 95 % CI) and post-partum support (p value= 0.032 , 95 % CI) were statistically significant in predicting post-partum depression while relationship assessment score (p value= 0.742 , 95 % CI) was not significant predictor. Exponential B values showed that for every one unit increase in depression score increased post-partum depression 1.223 times and for every woman who did not receive post-partum support, chances of developing postpartum depression were 3.9494 times higher than those who received support postpartum (Table 3).

4. DISCUSSION

This cross-sectional study carried out depicts that a higher rate of postpartum depression could be possibly due to various factors including bad relationship status, abusive marriages, family history of depression, history of miscarriages, gestational age, no family support postpartum, breastfeeding status, no. of children, employment status, family system, physical activities, and history of health issues among women. Our findings are consistent with previously reported studies [8, 31, 32].

The survey analysis aided in the examination of various factors such as depression scores, Relationship assessment scores, support received postpartum, and the probable cause of postpartum depression; family, health, or income problems were the main causes of postpartum depression. The majority of the women who were predicted to be positive for PPD had ages ranging between 30-40 years and the mean marrying age of the women who responded was 24.1942 (SD=3.4699). Most of the women claimed to have experienced postpartum depression with the birth of their first child. 80 % were university-level literate, 64.28 %

Table 3. Significant p-values (<0.000) indicate that Postpartum Support and Depression Score are significant contributors of postpartum depression

	B	Exp (B)	Significant value
Postpartum Support	1.223	3.494	.034
Depression Score	.201	1.223	.000
Relationship Assessment Score	0.004	1.004	.941

were non-working women; 65.97 % of the women experiencing post-partum depression lived in urban areas while 100 % of women in rural areas were predicted to be positive for postpartum depression. About 47 % of the women in the joint family system experienced PPD, whereas, 53 % of the women having nuclear family systems had PPD.

Prevalence of PPD among Pakistani women has been reported at 28-94 % [7, 8, 11, 33]. We investigated that PPD is highly prevalent in Pakistani women with 67.96 % (95 % CI). However, the frequency of PPD calculated in this study may not truly represent the actual incidence because of the small sample size. There could be multiple reasons for the elevated incidence of PPD in Pakistani women. Ideally, the postpartum period is considered a rest period for the new mother. She usually remains at home to recover from the birth for 40 days and is surrounded by family and friends during this time. Her extended family helps to take care of the home and any other children [13, 31]. If the woman perceived herself as not coping well or as not being able to care for her baby or her husband, she felt guilt and reported low self-esteem as she is expected to perform household chores, look after the children, and obey her husband and family diligently. Such women having lesser coping capabilities usually have a lack of care during the postpartum period. This may even have adverse consequences on the children along with the mother causing problems in the proper development of the baby [1, 34]. However, the adapted relationship assessment tool having lesser reliability did not turn out to be a good predictor of postpartum depression based on the relationship status of the women with their spouses.

Despite such higher rates of postpartum depression among Pakistani women, the issue is still neglected. It is the need of the hour to carry out more research to determine the important factors involved in the development and aggravation of PPD so that proper diagnosis, treatment, and preventative measures could be offered to the mothers which can help in the development of healthy mother-child bond and good family relations.

5. CONCLUSION

The outcomes of PPD on the mother and the child are devastating. The prevalence of PPD is much

higher than expected in Pakistan. For a more detailed analysis, more participants should be recruited from Pakistan to understand the exact frequency of this condition. Many treatment and management options can opt upon diagnosis. National-level screening setups should be established with the help of clinicians, psychologists, and researchers to carry out research and develop awareness among women regarding PPD.

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Exploring the Antimicrobials Production Potential of Actinobacteria Isolated from Caves at Bahadurkhel Karak, Pakistan

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Abstract: Cave actinobacteria are relatively less explored and are considered as the new targets for the discovery of novel antimicrobials, specifically against highly resistant pathogens such as extensively drug-resistant (XDR) *Salmonella*. In this study N=20 actinobacteria isolates were retrieved from the least disturbed caves situated at Bahadurkhel Karak, Pakistan (33° 16' 52" North, 70° 79' 07" East) and (33° 15' 90" North, 70° 94' 72" East). The isolates were identified using standard morphological and biochemical characterization procedures as well as by 16S rDNA sequencing. N= 16 isolates were targeted for further antimicrobial activity screening based on their preliminary morphological and biochemical identification. The methanolic extracts were prepared from the culture broth of the isolates by using Amberlite® XAD-16 resin to execute *in situ* solid phase extraction. The thin layer chromatography (TLC) showed the presence of a variety of different compounds in the crude extracts of the secondary metabolites. The extracts' antimicrobial potential was analyzed against several test strains, including *Salmonella enterica*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, and *Escherichia coli*. Among the isolated strains, the isolates SNK 93 and SNK47 were found to be the most active against the tested pathogenic bacteria, showing growth inhibition zones of 20 mm and 27 mm respectively against extensively drug-resistant (XDR) *Salmonella*. Based on these results it can be concluded that cave actinobacteria are a promising source of potentially novel antimicrobials against multidrug-resistant (MDR) and XDR pathogens.

Keywords: Cave Actinobacteria, Metabolites, XDR *Salmonella*, TLC, Antibiotics, Bahadurkhel Cave

1. INTRODUCTION

Known for the high G+C ratio in their genome, actinobacteria are indeed one of the largest groups of Gram-positive bacteria. They are generally regarded as potent producers of antibiotics, antifungal, anti-cancerous, and other bioactive compounds. More than 60 % of a current lot of antibiotics are being produced by the actinobacterial species, predominantly by the genus streptomycetes [1-4]. A chamber ideal to fit a human entrance could be termed as a cave. There are various ways to classify caves, however predominantly well-known types of caves are limestone and calcium-carbonated caves [5]. Microbiome data of caves is usually unexplored and underrated. The caves provide a unique stressful habitat to bacteria and other microbes, so are now attracting microbiologists for microbiome studies and for

exploring the microbiota for pharmaceutically significant metabolites [6-8]. Actinobacteria are predominantly present in caves because they are prolific producers of bioactive compounds there is no surprise if novel and potent antimicrobials can be recovered from the caves actinobacteria. This can be a solution for the antibiotic resistance problem in general and XDR pathogenic strains in specific [9]. Recent literature also suggests that cave actinobacteria species have been recovered with either new compounds or new analogs of existing active compounds [10-14]. In this regard, one of the mentionable discoveries is from Italy where researchers have been able to isolate cervimycins, which showed very significant activities against MDR Staphylococci and Vancomycin-resistant Enterococci (VRE) [15].

In recent years a lethal surge in extensively drug-

resistant (XDR) typhoid fever has been reported in different parts of Pakistan. Initially, the majority of the cases were reported from Sindh province in the districts of Hyderabad and Karachi [16]. Typhoid fever is caused by *Salmonella typhi*, [17]. The XDR *Salmonella* strains are resistant to a list of antibiotics that are routinely used to treat typhoid fever, including ampicillin, chloramphenicol, and trimethoprim-Sulfamethoxazole, ciprofloxacin, and ceftriaxone [18]. The situation has become alarming because the XDR *Salmonella* strains have now spread in the entire country and are causing high morbidity and mortality. Many advanced countries such as the United States, Canada, and the United Kingdom, etc, have reported the detection of XDR *Salmonella* cases and unfortunately, the majority of these cases had a travel history to Pakistan [19-23].

In this study we isolated actinobacteria strains from previously unexplored calcareous selective caves situated at Bahadurkhel district Karak, Pakistan, to screen them against a variety of pathogens including XDR *Salmonella*. The caves are located in a remote place away from the activities of native inhabitants, hence are the least disturbed caves. The idea of selecting these caves was based on the fact that these caves possess a unique stressed environment hence there are chances of getting new antimicrobials. As the caves can be harboring unique actinobacteria strains, this means that these strains could be potentially producing novel bioactive compounds including antibiotics. This study showed that actinobacteria isolates have the capacity to inhibit the growth of pathogenic strains including XDR *Salmonella*. This proves that cave actinobacteria are a unique source and need to be explored continuously for pharmaceutically significant compounds.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples (N=10) were collected aseptically from the ground, ceiling, and walls of caves. The caves are located in Bahadurkhel district Karak (33° 16' 52" North, 70° 79' 07" East) and (33° 15' 90" North, 70° 94' 72" East). The precise location of the sampling site is shown in Fig 1a, b. The caves are named locally as Kam Dadghor (KDG) and Star

Dadghor (SDG) respectively. The soil was dug for about 10-20 cm to avoid surface contaminants. The samples were aseptically transferred to the lab in sterile bags (Fig 2a). Extra care was taken to avoid light exposure by wrapping the sample boxes in black layerings.

2.2 Isolation of Cave Actinobacteria

A variety of selective media was used for the isolation in order to make sure to retrieve the maximum possible actinobacteria strains present in the sample. The Starch Casein Agar (SCA), Humic acid Vitamin Agar (HVA), Glucose Yeast Extract, Malt Extract (GYM), Oatmeal Agar (OM) and A medium were used to obtain diverse actinobacteria strains [24-26]. Among these media, starch Casein agar was used for both selective and pure culture isolation. The starch casein agar was simplified and modified in this study, as shown in supplementary data (Table S1). The modification and simplification involved the removal of trace salts to expose actinobacterial strains to a stressful environment which can potentially trigger their secondary metabolites production. Following isolation protocol was followed; 1 g of the soil sample was diluted in 10 mL of autoclaved normal saline which was then serially diluted up to 10⁻⁴ dilutions. 100 µL from 10⁻⁴ dilution was then spread onto SCA medium for initial isolation, after initial isolation, the rest of the media were used for obtaining the pure cultures. The putative actinobacteria isolates were streaked and maintained on the respective media.

2.3 Culture and Morphological Characterization

The putative actinobacteria strains were identified following a polyphasic taxonomic approach. First, the strains were randomly picked based on their morphology from a crowded plate, the criteria for picking up the actinobacteria strain was their apparent morphology and embedded growth in the medium and the colonies were examined for any sporulation if present [27]. Table 1 summarizes all the details of the characteristics which were considered and observed. Later all the isolates were characterized microscopically via gram staining and for biochemical characteristics (Fig 2b).

2.4 Biochemical Characterization

The isolates were also analyzed biochemically and were screened for melanin formation, organic acid formation and esculin hydrolysis. In the case of the organic acid production test, purple coloration was considered as positive, for melanin, and brown to black color was rendered as positive and for esculin hydrolysis, black coloration was noted as a positive result.

2.5 Preparation of Crude Extracts

To get secondary metabolites, the isolates were inoculated into 10 mL of SC broth and kept at 29 °C at 200 rpm for three days. After this enrichment step, 2 mL from this pre-culture was added to 50 mL of SC broth. Again this 50 mL culture was placed in a shaking incubator at 29 °C and 200 rpm for 8 days. About 1 % of Amberlite® XAD-16 N resin was then added to the fermented broth. The mixture was then sonicated for 20 m at 30 kHz and agitated for another 24 h under the same conditions and cellular mass was removed through centrifugation and methanol was used for eluting the active compounds from XAD resin, which was then evaporated on a rotary evaporator at 45 °C and weighed as shown in supplementary data (Table S2). The final product (crude extracts) obtained was kept at -20 °C for further usage and analysis.

2.6 Thin Layer Chromatography (TLC)

The metabolomic extracts were dissolved in Methanol, 5 mg of each extract was dissolved in 1 mL of HPLC grade methanol to obtain a solution of 5 mg/mL and the dissolved extracts were run on (20 × 20 cm Merck-Silica with Aluminum base TLC plates, thickness (20 μm) with binder Polymeric fluorescent indicator) [29]. Various mobile phases with different ratios were used to achieve maximum separation, based upon these trials, the optimum solvent system was found to be a 3:1 chloroform-methanol solvent system.

2.7 Antimicrobial Assay

The inhibition zones for the extracts were measured by the disc diffusion method against the following selected test strains. The first suspensions for the pathogenic test strains were prepared in Muller Hinton Broth (MHB) which included *Salmonella enterica*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, and *E. coli*. After the bacterial suspensions were incubated at 37 °C for 24 hours. The optical density for the tested strains was adjusted at 0.5 (10⁸ CFU/mL) Mcfarland standards. Crude Extracts of about 30 mg were dissolved in 250 μL of methanol, and 25 μL which is equal to 3 mg of the extracts were loaded onto the sterile discs (6 mm), which were placed in the pre-inoculated plates with test strains [30]. The test strains were spread onto the MH agar with the help

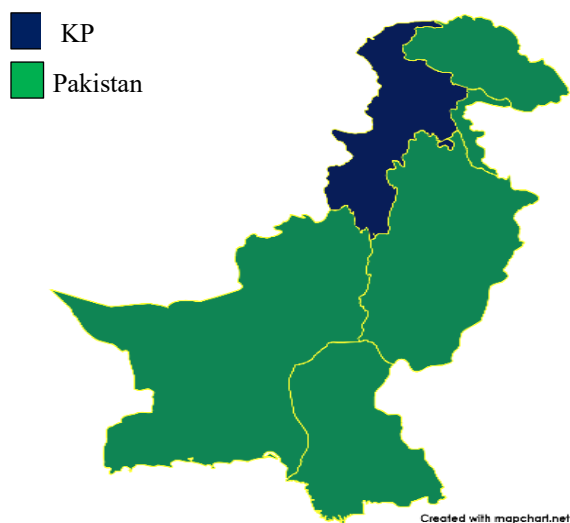
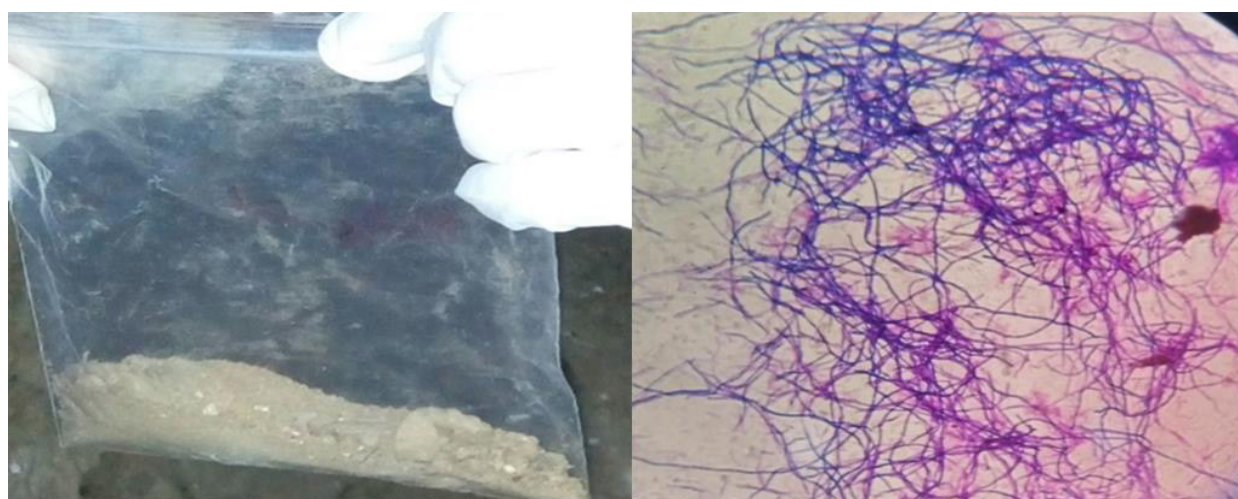


Fig. 1. (a) Overall indication of the caves from a generalized map, (b) Zoomed Map of Bahadurkhel showing specific sampling sites

Table 1. Morphological characteristics of Actinobacteria isolates from caves

Isolates	Colony Morphology	Substrate Mycelium	Aerial Mycelium	Pigments
SNK47	Embedded	Yellow	greyish yellow	white
SNK35	Rough	-	white	white
SNK59	Rough	Grey	white	white
SNK 220	Embedded	Grey	grey	white
SNK04	powdery	White	white	white
SNK03	Rough	Grey	white	white
SNK 73	Powdery	Yellow	green	green
SNK11	Embedded	White	white	white
SNK 250	Embedded	-	white	white
SNK 95	Embedded	Pink	pink	pinkish white
SNK 242	Embedded	-	white	white
SNK246	embedded	-	white	white
SNK106	embedded	White	Grey	white
SNK25	embedded	Grey	Grey	white
SNK 80	embedded	Grey	white	Grey
SNK 93	embedded	-	white	Grey

**Fig. 2.** (a) A soil sample collected from the SDG cave of Bahadurkhel Karak, (b) Gram staining of strain SNK 47

of sterile cotton swabs to prepare a uniform lawn of the test bacteria. The 25 μ L of methanol was also added in an empty well as a negative control.

2.8 Extraction of Genomic DNA

The genomic DNA was extracted using Thermo Scientific Gene JET Genomic DNA purification Kit #K0721 (Lithuania) in accordance with the manufacturer's instructions. Briefly, the samples were lysed with proteinase k in lysis buffer and RNA was removed using RNase A, after that the lysate was mixed with ethanol and by using purification

columns and washing solutions all the impurities were removed before the genomic DNA was eluted in elution buffer and the DNA was stored at -20 $^{\circ}$ C for further use.

2.9 Polymerase Chain Reaction for The Amplification of 16S rDNA

The polymerase chain Reaction for the extracted genomic DNA using universal primers 27F 5'- AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-CTACGGCTACCTTGTTACGA-3' was performed maintaining the following cyclic

conditions. Denaturation at 95 °C for 5 mins followed by 30x cycles of denaturation at 94 °C for 30 sec, annealing at 61 °C for 30 sec, and extension at 72 °C for 30 sec. The final extension step was set at 72 °C for 5 mins followed by an infinite holding time at 4 °C. The purified PCR products were sequenced using Automated Sanger chain termination sequencing.

3. RESULTS AND DISCUSSION

3.1 Morphological Identification through Gram Staining and Biochemical Characterization

All the strains from the three culturing media were streaked onto 5 isolation media, including Starch Casein Agar, A medium and glucose yeast extract malt extract media. Among all the starch Casein agar was found to be the most suitable isolation media. We modified and simplified the starch casein agar media. The recipe for the modified media is given in supplementary data (Table S1). For morphological identification, the isolates were characterized based on the texture of the colony and their pattern of growth on the agar surface, as generally, actinobacteria colonies show embedded growth into the agar medium (Fig. 3). Other than these aspects pigment formation is also a very important classification and identification tool to isolate actinobacterial strains, in this study, it was

found that Green and yellow were the predominant pigments produced by the caves actinobacteria isolates. SNK 47 and SNK 93 were strongly positive for melanin formation and organic acid formation, SNK03 and SNK 04 were found to be producing a good amount of organic acid. Overall results of the tests are summarized in Table 2.

3.2 Thin Layer Chromatography Profile of the Extracts

The compounds in each of the sample got separated based on their relative polarity. The selected isolates were chosen based on their TLC chromatogram for metabolomics profiling. The chromatogram was analyzed under UV at 254 nm and 366 nm using CAMAG® Uv Cabinet 4 Switzerland. Alternatively, the extracts were also observed by staining with Anisaldehyde (Fig 4). SNK 47 and SNK 93 showed a distinct pattern of bands suggesting the presence of a variety of metabolites and compounds, based upon these results the extracts were tested against a variety of pathogens including XDR *Salmonella*.

3.3 Antimicrobial Activity of the Metabolites against Test Strains

Among all the strains, the isolates SNK 47 and SNK 93 were active against all the tested strains.

Table 2. Summary of biochemical characteristics of cave actinobacteria

Isolates	Biochemical Tests		
	Melanin Formation	Esculin Hydrolysis	Organic Acid Formation
SNK47	+	+	+
SNK 93	+	+	+
SNK 35	+	+	-
SNK 03	+	+	+
SNK 220	+	+	+
SNK 73	+	+	-
SNK 04	+	+	+
SNK 11	+	+	-
SNK 250	-	+	-
SNK 242	+	+	+
SNK 246	-	+	+
SNK 25	+	+	+
SNK 106	+	+	+
SNK 95	+	+	+
SNK 59	-	+	+

Keys: + = positive for the test, - = negative for the test

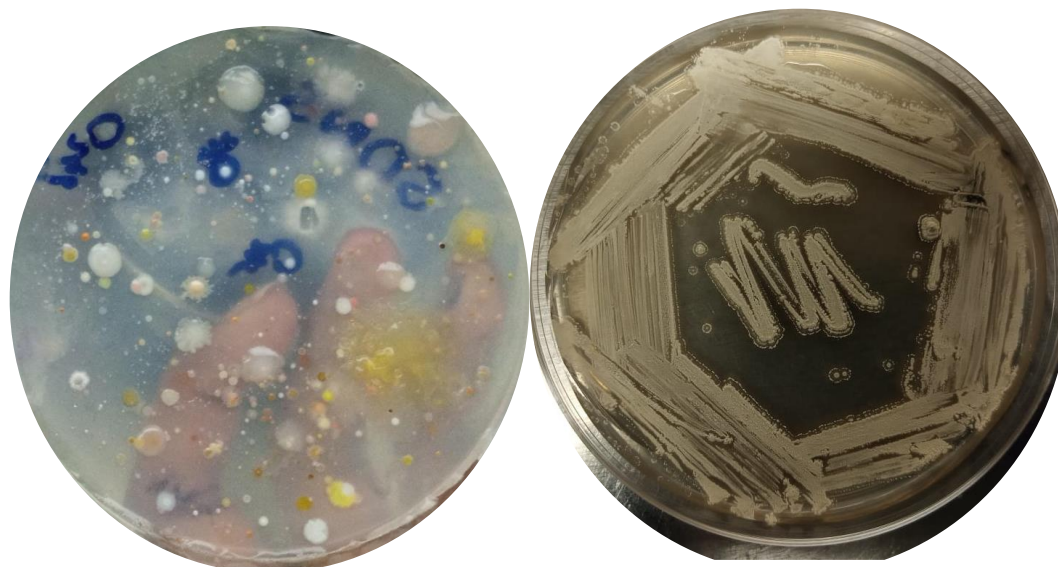


Fig. 3. (a) A crowded plate, showing putative actinobacterial isolates, (b) pure culture of SNK93 on starch casein agar

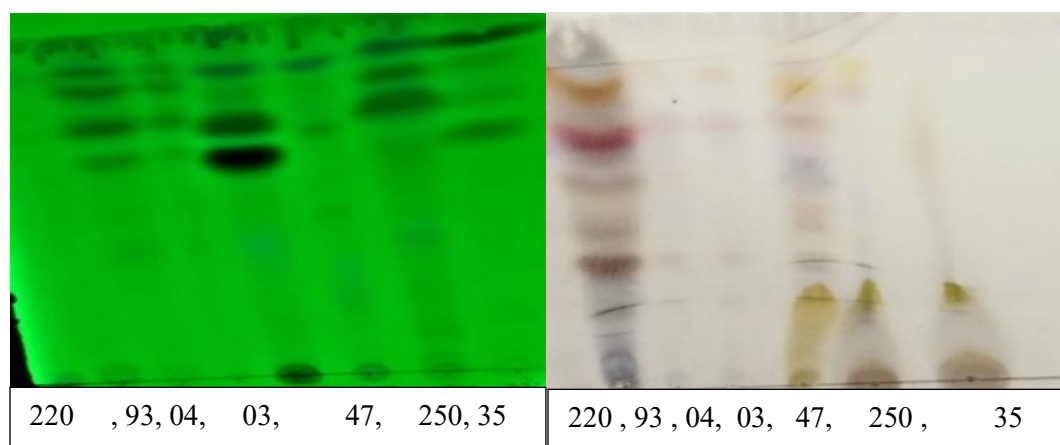


Fig. 4. (a) TLC chromatogram at UV 364 nm, showing separation of compounds based on relative charge and polarity (b) staining with Anisaldehyde/H₂SO₄

However, the activity of SNK 47 was found to be maximum against XDR *Salmonella* (Table 3). The XDR *Salmonella* was isolated from meat samples. The zones of inhibition measured for the SNK 47 against *Salmonella*, *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumonia* and *Bacillus subtilis* were 27 mm 12 mm, 11 mm 10 mm and 10 mm respectively. While for SNK93 the zones of inhibition were measured as 20, 0, 0, 10 and 09 mm respectively (Fig 5a). After initial scrutiny of all the crude extracts against XDR *Salmonella*, it was found that only SNK 93 and SNK 47 were active against XDR *Salmonella*. That is why both of the extracts were then used in replicates against XDR

Salmonella to ascertain the results. Both of the extracts from the said isolates produced comparable results in replicates (Fig 5b).

The isolates SNK 04 and SNK 35 showed some activity against *Bacillus subtilis*. The zone of inhibition for these isolates were measured at 10, and 12 mm respectively. In general SNK 47 was found to be the most active strain as it showed 100 % activity against all the test strains showing that it possesses the remarkable potential for antibiotics production which can be used against different pathogens including XDR *Salmonella*. However, SNK 93 was found to be active against

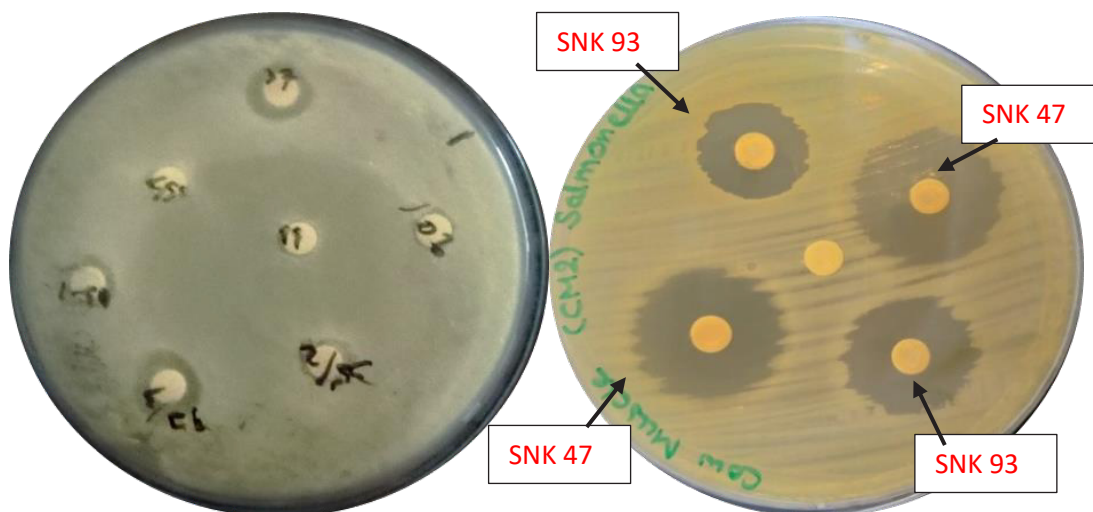


Fig. 5. (a) Antimicrobial activity of the methanolic crude extracts against *Bacillus subtilis*, activities of SNK 47, 93, 04, 106 and 35. **(b)** Activities of the crude extracts of SNK 47 and SNK 93 against XDR *Salmonella*. Both extracts were tested in replicates against XDR *Salmonella*.

Table 3. Zone of inhibition recorded in Millimeters against Test strains

Isolates	zone of inhibition recorded in mm against test strains				
	<i>Salmonella</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Bacillus subtilis</i>
SNK47	27	12	11	14	15
SNK 93	20	0	0	10	9
SNK 35	0	7	0	7	10
SNK 03	0	7	0	0	9
SNK 220	0	0	0	0	0
SNK 73	0	0	0	9	12
SNK 04	0	0	0	0	15
SNK 11	0	0	9	0	14
SNK 250	0	7	8	0	8
SNK 242	0	8	13	0	11
SNK 246	0	10	11	0	10
SNK 25	0	8	10	7	13
SNK 322	0	0	10	0	9
SNK 95	0	8	0	7	9
SNK 59	0	0	9	8	10

three test strains showing an overall 60 % activity against all the test strains. These results suggest that SNK 47 and SNK 93 can further be exploited for antibiotics production.

3.4 16S rRNA gene Sequencing and Phylogenetic Analysis

The sequencing data was analyzed through Finch TV 1.4. The nucleotide sequence was then BLAST analyzed to determine the similarity against the existing data of *actinobacteria* in NCBI [31]. The

results for SNK 47 showed that the strain is 99 % similar to that of *Glutamibacter uratoxydans*, For the SNK93 the similarity index was found to be 99 % against *Actinoplanes* sp. SE50. Both of these isolates are categorized as rare actinomycetes. The neighbor-joining phylogenetic tree for both SNK 47 and SNK 93 is given in figures 6 and 7. The evolutionary history was inferred using the Neighbor-Joining method, the evolutionary distances were computed using the Maximum Composite Likelihood method and in the units of the number of base substitutions per site. The analysis

for SNK 93 involved 6 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 593 positions in the final dataset. The analysis for SNK47 Involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 322 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Due to their unique habitat, Caves provide one of the best competing environments to microbes where they can produce a variety of compounds and secondary metabolites to survive [32-34]. Cave actinobacteria in this study have been primarily targeted to assess Their potential against a variety of pathogens including XDR *Salmonella*. A variety of innovative methods were executed to obtain the optimum number of actinobacterial isolates. One of these methods includes simplification and modification of the Starch Casein agar Media given in Supplementary data (Table S1).

The trace salts were removed from the media and pH was adjusted to 7.8 so that the cave microbes could experience a stressful environment and hence this may activate their secondary metabolites for survival. The media showed perfect growth for cave actinobacteria. Antibiotic resistance is a serious concern in recent days, as WHO estimates that there will be no treatment options in the future if the rate of resistance among the bacteria continues

to be the same [35, 36]. The problem is that the rate of resistance in bacteria is much more than the discovery of novel antibiotics. One possibility is to reduce the use of antibiotics and find alternatives but there is no significant progress in this regard. Medical world will rely on antibiotics in the future as well. Generally acceptable solution in this regard is to find a new antibiotic or new version (analogs) of existing antibiotics which can inhibit infectious bacterial growth [37, 38]. One promising and classic source in this regard is actinobacterial species which are producing more than 60 % antibiotics. However, actinobacteria from the most common sources such as soil have been highly exploited and there is less hope of finding any novel antibiotic agents from these sources. Recent studies have shown that Caves are one of the most unexplored habitats having unique environmental stress which can trigger the production of novel and potent antibiotics [39]. In this study among the 15 isolates, 4 isolates showed promising activity against a variety of pathogens including XDR *Salmonella*. Specifically, the isolates SNK 47 and SNK 93 were found to be highly active against XDR *Salmonella* and the rest of the pathogens. This shows that caves can be a good source of finding new and active metabolites against MDR and XDR pathogens. Sequencing analysis showed that the SNK 47 and SNK 93 are 99 % of that of *Glutamibacter uratoxydans* and *Actinoplanes* sp. SE50. To the best of our knowledge, this could be the first report of isolating *Glutamibacter uratoxydans* from the cave environment. *Actinoplanes* spp, however previously

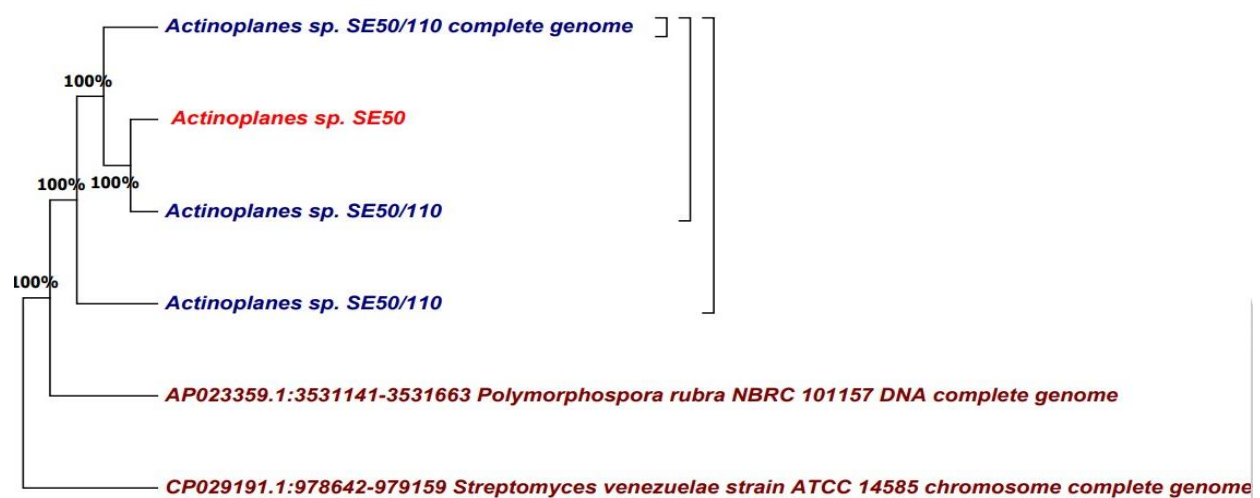


Fig. 6. Phylogenetic tree showing the relationship of SNK 93 to the *Actinoplthe anes* (Highlighted in red). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

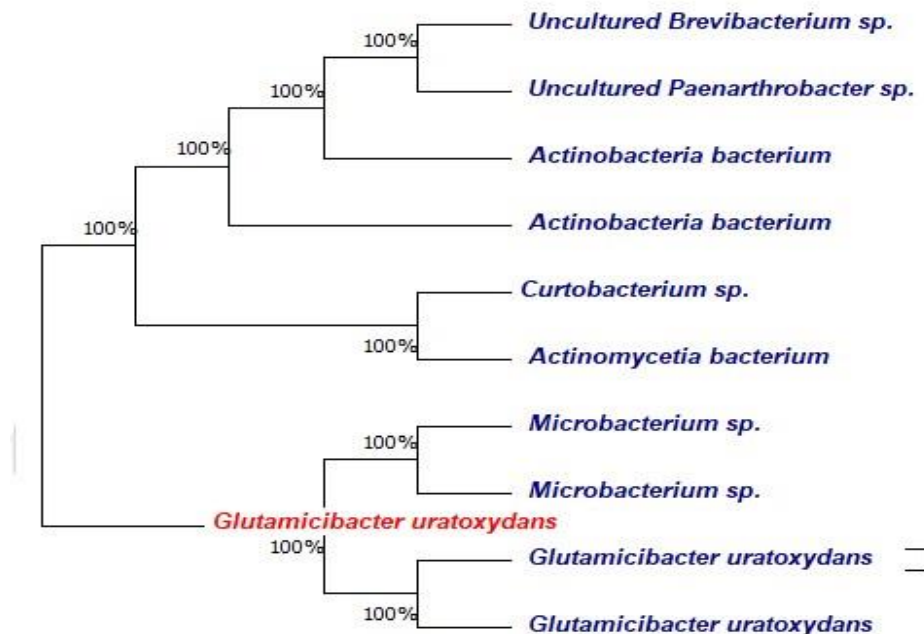


Fig. 7. Phylogenetic relationship of SNK 47 with *Glutamibacter* spp (highlighted in red). The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. This analysis involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 322 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

have been isolated from caves [40]. This study will further be extended to identify these metabolites and also determine the structures of these active compounds. Classical approach in this regard is large-scale cultivation, purification of compounds through HPLC and structure elucidation through LC-MS and NMR methodology. The study can be used as a benchmark study in exploring other caves in the region for a large-scale hunt of potent bioactive compounds and novel antibiotics. Other alternative goals that can be achieved by exploiting caves are the discovery of some novel actinobacterial genera as well as their subsequent metabolite profiling which may result in the discovery of many other novel compounds such as antitumor, antifungal and agro active compounds other than antibiotics.

4. CONCLUSION

The actinobacteria isolates from caves showed promising activities against pathogens including XDR *Salmonella*, which supports the idea that caves can be a good source of finding novel and potent antimicrobial agents. The strains SNK47 and SNK 93 are the most active isolates. Targeting these Isolates for Whole genome sequencing, Molecular

networking coupled with traditional compound isolation and purification protocols may lead us toward the discovery of novel antibiotics and other bioactive compounds from these strains. There is a need to extensively analyze caves and large-scale studies may help find some of the very promising antibiotics and other bioactive compounds.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

7. DECLARATION

This study did not use any animal models or human subjects.

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SUPPLEMENTARY DATA

Table S1. Modified Starch Casein Agar

Media Components	G/L
starch	10
casein	1
calcium carbonate	2
Agar	13

Table S2. Weight of crude extracts

Isolate	Weight (mg)
SNK47	42
SNK 93	31
SNK 35	29.9
SNK 03	40.4
SNK 220	50
SNK 73	33.5
SNK 04	34.2
SNK 95	25.7
SNK 250	47
SNK 242	33
SNK 11	39
SNK 246	33
SNK 25	45
SNK 106	37
SNK 59	48



Photodegradation of Direct Violet 51 Dye using $\text{Bi}_2\text{MoO}_6/\text{GO}$ Nanoflakes as Promising Solar Light-driven Photocatalyst

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Abstract: Water contamination is a challenging issue for the maintenance of environmental sustainability. Industrial effluents are considered major sources of water pollution which affect the quality of surface as well as ground water. In the present research work, semiconducting Bismuth Molybdate/Graphene Oxide ($\text{Bi}_2\text{MoO}_6/\text{GO}$) composite nanomaterial has been introduced as the solar light-driven catalyst for photodegradation of Direct Violet (DV) 51 dye and industrial wastewater. Scanning electron microscope (SEM), zeta potential, X-ray diffraction analysis and Fourier transform infrared spectroscopy (FTIR) were used to characterize the $\text{Bi}_2\text{MoO}_6/\text{GO}$ composite material. Experimental findings revealed that flake-like $\text{Bi}_2\text{MoO}_6/\text{GO}$ composite exhibits 99.00 % degradation activity against DV dye within 80 minutes. $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes degrade DV dye up to 98.70 % at pH 7 and 99.99 % with a 100 mg catalyst dose within 60 minutes, respectively. The stability/reusability study presented 99.82 % - 93.84 % dye degradation from the 1st to 7th day within 80 minutes while optimizing experimental parameters. According to kinetic studies of experimental outcomes, the pseudo-first-order model was best fitted to the obtained data with a coefficient of determination $R^2=0.954$. Moreover, a 69.23 % reduction was observed in chemical oxygen demand (COD) during the photodegradation study of industrial wastewater. Results indicate that $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes have good photocatalytic potential and stability to degrade organic water pollutants under sunlight. Such materials can be used effectively for the photodegradation of organic water pollutants to enhance environmental safety.

Keywords: Water pollution, Direct violet 51 dye, Photodegradation, Graphene oxide (GO), Bismuth molybdate (Bi_2MoO_6).

1. INTRODUCTION

Water pollution has become a severe ecological threat globally, it requires due attention to maintain a healthy lifestyle. There are a variety of organic pollutants like dyes, pesticides, fungicides, drugs, and food toxins in environmental components such as soil, water, crops, vegetables, fruits, and juices, etc. which exert undesirable effects on the human as well as other living creatures [1]. Their release into water systems badly disturbs the quality of both ground and surface water leading to critical health issues in living beings. Due to their non-

biodegradable nature and long-range transport, they accumulate in living tissues and disturb the food chain [2]. Among the released waste products into water bodies, dyes are considered as coloring materials having potential applications in multiple fields like textile, leather, plastics, paper, precious stones, food products, etc. The addition of such complex organic compounds into water bodies inhibits solar light transmission and affects aquatic life. On average there are about more than one hundred thousand synthetic dyes having an estimated production of seven hundred thousand tons/annum throughout the world, resulting in

substantial production of wastewater [3].

Direct violet 51 dye is found in the class of direct dyes including water-soluble anionic azo dyes. According to the Santa Cruz Biotechnology, Inc. supplier's safety data sheet, Direct violet 51 is a synthetic, anionic dye having double azo groups. These azo groups act as chromophores to impart color in the textile, paper, and leather industries. Metabolism of azo groups prone to produce carcinogenic aromatic amines such as benzidine. Complex direct dyes have more than one azo linkage and their ingestion or contact may lead to mutagenic, cardiogenic, chronic bronchitis, vomiting, nausea as well as skin, eye, and respiratory tract irritation in humans. In aquatic media, they block the penetration of sunlight and interrupt the photosynthesis in aquatic plants leading to disturb marine life [4].

Removal of such toxic pollutants is a critical situation for the environment and the agencies related to it. A lot of work has been conducted in this area using conventional techniques like filtration, sedimentation, coagulation, membrane separation, flocculation, and adsorption, etc. No doubt these techniques have their importance but also have some limitations i.e., laborious, time taking, high consumption of chemical reagents and transform the pollutants from one phase to another by generating secondary pollutants which result in incomplete removal of toxic substances [2]. Among these progressions, heterogeneous photocatalysis has attracted much attention as a facile and cost-effective method. It is a well-known advanced oxidation method that utilizes light-sensitive photocatalysts to accelerate photochemical reactions by harvesting solar energy and results in the complete degradation of organic pollutants without generating secondary pollutants [5, 6].

Growing complications in environmental pollution draw considerable attention to searching nanomaterials bearing multiple catalytic potentials. Their significance increases due to the highly efficient mode of action and cost-effectiveness than conventional techniques [7]. Semiconducting metal oxides and their composites with carbon-based nanostructures have fascinating photocatalytic applications due to their synergistic effects. Two-dimensional (2D) materials (nanosheets, nanoplates, nanoflakes, etc.) play a major role in

photocatalytic applications due to their outstanding properties, offering large interlayer spaces and high active surface area owing to more exposed surface atoms. Their flattened and thinner dimensions provide multiple pathways for the migration of charge carriers augmenting the photocatalytic potential at the atomic level [8].

2D Graphene oxide (GO) has outstanding electrochemical characteristics due to its electron promotor attributes. The presence of reactive oxygenated functional groups provides stable chemical reactivity as a catalyst as well as for the formation of composite materials with other metallic nanostructures to overcome the recombination of charge carriers during photocatalysis [9]. Bismuth molybdate (Bi_2MoO_6) belongs to the family of Aurivillius oxide perovskites. Bi_2MoO_6 is a very stable semiconductor material offering good photocatalytic activity due to its perovskite layered structure. Its crystal structure has corner-sharing MoO_6^{2-} octahedrons sandwiched between alternating $\text{Bi}_2\text{O}_2^{2+}$ layers providing a large active surface area for the effective separation of charge carriers [10, 11].

In the present study, we introduced the synthesis and application of Bi_2MoO_6 /GO nanoflakes. GO was synthesized by Hummer's method while Bi_2MoO_6 /GO composite nanoflakes were developed through a hydrothermal route. The synthesized Bi_2MoO_6 /GO composite nanoflakes were applied as solar light-driven nano-photocatalysts to degrade Direct violet 51 dye, a model organic water pollutant. To study the real-life application of these nano-photocatalysts, industrial wastewater was also treated with composite nanoflakes under sunlight. Experimental outcomes revealed excellent photodegradation efficiency of Bi_2MoO_6 /GO nanoflakes against model dye and industrial wastewater by harvesting solar energy. According to the author's knowledge, there is no elaborated work on the photodegradation of direct violet 51 dye by using Bi_2MoO_6 /GO composite as a photocatalyst.

2. MATERIALS AND METHODS

2.1 Chemicals/Reagents

Chemicals and reagents i.e. hydrogen peroxide (H_2O_2 , 35 %, BDH), graphite powder

(98.0 % UNI-CHEM), sodium hydroxide (NaOH, 98-100 %, Sigma Aldrich), sulphuric acid (H_2SO_4 , 98 %, Merck), methanol (99.5 %), ethanol (99.8 %), sodium molybdate dihydrate ($\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, ≥ 99.5 %), acetic acid (CH_3COOH , 99 %, Merck), bismuth nitrate pentahydrate [$\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$, 98 % Merck], potassium permanganate (KMnO_4 , 99 %, RFCL) hydrochloric acid (HCl , 36.5-38 %, Sigma Aldrich), and sodium nitrate (NaNO_3 , ≥ 99.0 %, Merck) were used as such without any treatment. Deionized water (DI) was used as a solvent for the preparation of solutions obtained from the Millipore-MilliQ system.

2.2 Production of Graphene oxide (GO)

Modified Hummer's method was used to synthesize graphene oxide (GO) by the oxidation of graphite [12]. In a typical synthetic route graphite powder and sodium nitrate were added in a 6:3 ratio to the concentrated sulphuric acid (200 mL) in the ice bath at constant stirring. After 25 minutes potassium permanganate (40 g) was added slowly on vigorous stirring by maintaining the solution temperature below 15 °C for 2 hours. To obtain a brownish dense slurry the reaction media was kept on constant stirring at 30-35 °C for 36-48 hours. Then deionized water was added slowly, temperature of the reaction medium was raised to 90 °C and maintained at this temperature for about 25-30 minutes. The suspension was cooled down at room temperature and 60 mL H_2O_2 was added dropwise on constant stirring to stop the reaction. After 10 minutes, 10 percent HCl was added to the suspension and allowed to settle down brownish GO precipitates for 24 hours, GO precipitates were collected, methanol, and deionized water were used as washing solvents to eliminate the impurities from the final product and desiccated at 60 °C under vacuum.

2.3 Synthesis of Bismuth Molybdate graphene oxide (Bi_2MoO_6)/GO composite nanoflakes

A facile hydrothermal method was used to synthesize (Bi_2MoO_6)/GO nanoflakes. For this, a 40 mL sodium molybdate dihydrate ($\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$) solution (0.07 M) was prepared in DI water termed as (A). Meanwhile, 20 mL 0.1M bismuth nitrate pentahydrate was dissolved in 2.5 M acetic acid on constant stirring as the solution (B). Graphene oxide (GO) 40 mg was dispersed in 20 mL of

deionized water via sonication. Solution (A) was added to the solution (B) dropwise on continuous stirring. Consequently, graphene oxide suspension was added to the resulting suspension and left on continuous stirring for 30 minutes. Then this suspension was poured into a 200 mL stainless steel, Teflon-lined autoclave to place in a heating oven at 180 °C for 16 hours. After completion of the reaction time, the autoclave was cooled down at room temperature. (Bi_2MoO_6)/GO product was collected, washed with absolute ethanol, and deionized water to purify the material. The obtained material was dried in the heating oven at 80 °C for 6 hours. The dried material was ground to a fine powder and stored in an airtight jar.

2.4 Characterization

Scanning electron microscope Raith-e-Line was used to investigate the morphology of prepared nanostructures. For SEM analysis, the suspension was prepared by dispersing 1 mg of (Bi_2MoO_6)/GO material in 2 mL absolute ethanol via sonication. 20 μL suspension was spin-coated on carbon-coated copper grids and allowed to dry to obtain a stabilized layer. Phase purity and crystal structure were determined by X-ray diffraction technique. The spectra were recorded in the 2-theta range of 10-90 ° on Bruker D8 Discover X-ray Diffraction System with $\text{Cu K}\alpha$ radiation i.e. $\lambda=0.15406$. Zeta Sizer Nano Series from Malvern Instruments was used to estimate the zeta potential/surface charge of the as synthesized (Bi_2MoO_6)/GO material. To determine the active functional groups in the obtained composite, the material was analyzed through Fourier transform Infrared spectroscopy using Cary-630 Fourier transform Infrared spectroscope in ATR (attenuated total reflection) mode.

2.5 Application of Nanomaterials as Photocatalysts

To study the photocatalytic degradation activity of synthesized (Bi_2MoO_6)/GO nanostructures against model dye, solar light was used as an irradiation light source to accelerate the nanomaterials as photocatalysts. 1000 ppm stock solution of direct violet 51 (DV) dye was prepared by adding 0.1 g of DV in 1000 mL deionized water. Then further dilutions (10-100) ppm were prepared from the stock solution by using the dilution formula.

To see the effect of contact time and kinetic study 50 mg (Bi_2MoO_6)/GO nanostructures were added in 20 ppm, 50 mL Direct violet 51 (DV) dye solution and kept in dark for a half hour for maintaining adsorption and desorption equilibrium of dye molecules on the surface of nanostructures. Then subjected to solar light for different time intervals (0-80 minutes). The rate of dye degradation by nanostructures was determined by evaluating the decrease in the relative maximum absorbance at the characteristic wavelength ($\lambda_{\text{max}} = 586 \text{ nm}$) of the dye solution. For this, after regular time intervals 3 mL solution of direct violet 51 dye was taken out and centrifuged at 500 rpm to separate any suspended nanostructures and the intensity of relative absorbance at 586 nm was measured by UV/Visible spectrophotometer.

To study the effect of pH 0.1 M HNO_3 and 0.1 M NaOH solutions were utilized to adjust the pH of dye solution from 3-9. 20 mg of nanostructures were added in dye solutions having the same concentration (20 ppm), equal volume (20 mL) but different pH (3-9), and subjected to sunlight for 1 hour. To see the reusability and stability, (Bi_2MoO_6)/GO photocatalysts were used again and again to degrade the respective new dye solution of the same concentration. For this, the (Bi_2MoO_6)/GO nanostructures were collected by centrifugation from treated dye solutions, washed with ethanol and deionized water to remove any residual dye molecule, dry at 60-80 °C for 4-6 hours. After complete drying, the nanocatalysts were again added into the fresh dye solution of the same concentration (20 ppm) and equal volume (20 mL) under vigorous stirring in dark for a half hour and then subjected to solar light for respected time e.g. 60 minutes. The concentration of residual dye was observed by recording the difference in absorbance of respected maximum wavelength ($\lambda_{\text{max}} = 586 \text{ nm}$) through UV/Vis spectrophotometer to see the difference in % degradation. The percentage degradation of model dye was assessed through the given formula.

$$\% \text{ degradation} = \frac{C_0 - C_t}{C_0} \times 100$$

Here, “ C_0 ” denoted the initial dye concentration at time (t)=0, while “ C_t ” is the dye concentration after treatment with photocatalysts at a given time ($t = 10, 20, 30, 80$ minutes, etc.).

2.6 Chemical Oxygen Demand (COD)

For the real-life application of as synthesized nanostructures, industrial wastewater containing various dyes was treated with (Bi_2MoO_6)/GO composite. Chemical oxygen demand (COD) was calculated to check the degradation efficacy of nanostructures against organic matter in wastewater. [13]. Three working solutions were used during the experimental study of chemical oxygen demand. Digestion solution (70 mM Potassium dichromate), 200 ppm potassium hydrogen phthalate (KPH) standard solution, and 5 g $\text{AgSO}_4 / 500 \text{ mL}$ of Concentrated Sulphuric acid were used as catalyst solution.

Three test tubes were used in triplicate, e.g., three test tubes were marked as blank, three for standards and three for sample solution, for triplicate run respectively. Catalyst solution (2.8 Ml) and 1.2 mL of digestion solution were poured into all test tubes. For blank, 2 mL deionized water, 2 mL KPH as the standard solution, and 2 mL industrial wastewater as standard, and the sample was added in separate test tubes in triplicate, respectively. All test tubes were refluxed well to homogenize the mixtures and then placed in a heating reactor at 150 °C for two hours. After this, test tubes were cooled down at room temperature for 30 minutes, and recorded the absorbance of treated blank, standard, and sample solution was at a wavelength of 600 nm via spectrophotometer. The difference in chemical oxygen demand before and after treatment was calculated using the formula;

$$\text{Chemical oxygen demand} = \text{standard factor} \times \text{optical density}$$

$$\text{COD removal (\%)} = \frac{\text{Initial COD} - \text{Final COD}}{\text{Initial COD}} \times 100$$

3. RESULTS AND DISCUSSION

3.1 Possible Growth Mechanism of $\text{Bi}_2\text{MoO}_6 / \text{GO}$ Nanocomposite

During hydrothermal growth reactions, Bi_2MoO_6 crystals were formed in 2 steps (1) Bismuth ions (Bi^{3+}) interacted with water molecule/OH-groups and converted into the $\text{Bi}_2\text{O}_2^{2+}$ ionic layers. (2) $\text{Bi}_2\text{O}_2^{2+}$ ionic layers interact with MoO_4^{2-} ions

and are converted into Bi_2MoO_6 nuclei and then arranged into a specified flake-like shape according to the reaction conditions. Graphene oxide interacts with Bi_2MoO_6 nuclei through the electrostatic or hydrogen bonding forces leading to the formation of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanocomposite material. The presence of oxygenated functional groups in GO plays a major role in its chemical reactivity towards other nanostructures [14].

3.2 Morphology and Structural Analysis of $\text{Bi}_2\text{MoO}_6/\text{GO}$ Nanocomposite

Morphology of graphene oxide (GO) and $\text{Bi}_2\text{MoO}_6/\text{GO}$ composite was evaluated by scanning electron microscopy. Figure 1 (A) shows the flattened, thin sheet-like morphology of graphene oxide nanosheets. GO has crumpled, layered morphology. Ultra-thin GO sheets are rippled and entangled with each other having randomly oriented corners or edges. SEM analysis of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanomaterial shows the high yield of 2D flake-like morphology having different size and shape distribution. Nanoflakes have flattened surfaces with zigzag margins. It was also observed that the nanoflakes have one broader and other least broadened ends. Nanoflake's analysis through Image J. software revealed that they have a thickness of around 40-50 nm, width and height were observed to be more than 200 nm. The flattened surface plays a major role in increasing the active surface area of 2D nanomaterials as shown in Figure 1 (B).

Structural and functional characterization of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes is shown in Figure 2. The crystallinity and phase purity of the composite material was analyzed through a powder X-ray diffraction study. XRD analysis revealed the composition of nanoflakes as bismuth molybdate according to the given Empirical and Chemical formulae: Bi_2MoO_6 . All diffraction peaks in the XRD pattern were found to be well indexed to the orthorhombic crystalline structure with lattice parameters $a=5.5060$ (Å), $b=16.2260$ (Å), and $c=5.4870$ (Å) with JCPDS Card No. 01-072-1524.

The diffraction peaks were detected at 2-theta values of 11.1° , 14.5° , 19.6° , 26.5° , 28.5° , 32.5° , 35.2° , 46.71° , 51.15° , 52.31° , 55.40° , 58.44° , 75.66° , and 86.80° correspond to (020), (021), (040), (121), (131), (002), (022), (152), (023), (024), (262), (333) and (462) hkl planes of nanoflakes (Figure 2 A). The sharp and intense peaks illustrate that the sample is well crystallized even in composite form with GO. There was no specific peak for GO which might be due to less concentration of GO as compared to Bi_2MoO_6 . The superior relative intensity of the (131) diffraction peak at 28.5° exhibits the (131) preferred orientation in the $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes [15].

FTIR profile of the $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes shows the structural functionalities in Figure 2(B). The band that appears at 3468 cm^{-1} might be assigned to O-H bonds stretching vibrations. The

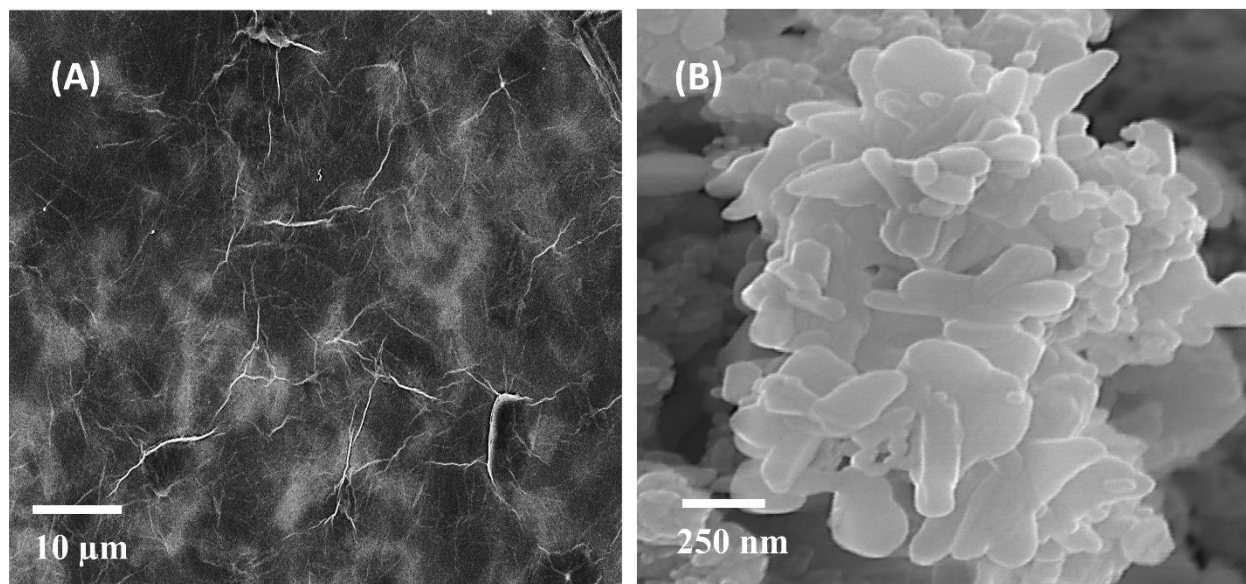


Fig. 1. SEM images showing morphological characterization of GO (A) and $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes (B).

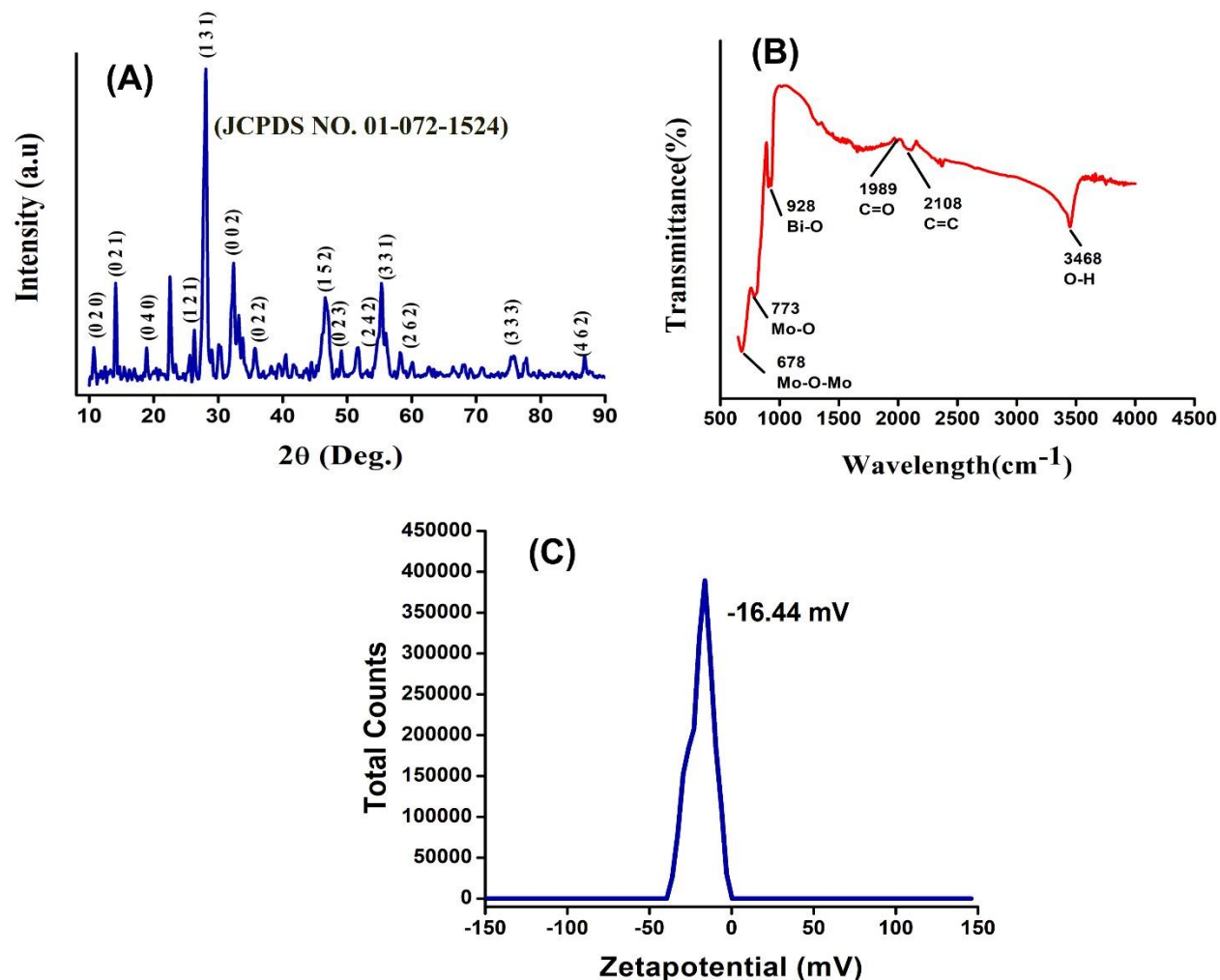


Fig. 2. Structural and functional characterization of Bi₂MoO₆/GO nanoflakes. XRD pattern displaying phase purity (A), FTIR Profile (B), Zeta surface potential scan (C).

two consecutive bands appearing at about 1988 and 2108 can be attributed to the C=O bending vibrations of carboxyl groups and aromatic C=C bending vibrations in GO respectively. The bands centered at 980 cm⁻¹ and 773 cm⁻¹ were assigned to the stretching vibrations of Bi-O and Mo-O respectively. Another band appearing at 678 cm⁻¹ was well indexed to bridging stretching vibrations of Mo-O-Mo bonds [15]. Figure 2(C) shows the zeta potential analysis of the Bi₂MoO₆/GO nanoflakes. The surface charge was found to be -16.44 mV revealing that the material is negatively charged. Surface charge plays an important role in the colloidal stability of the nanomaterial [16, 17].

3.3 Photocatalytic Degradation of Direct Violet (DV) dye by Bi₂MoO₆/GO nanoflakes

The solar light harvesting photocatalytic efficiency

of Bi₂MoO₆/GO nanoflakes was estimated against the photodegradation of Direct Violet (DV) dye as a model organic water pollutant. The absorption spectra of the under-test DV dye solution was recorded from 400-800 nm and the characteristic maximum absorbance was observed at 586 nm. Bi₂MoO₆ nanostructures are used as good photocatalysts for the degradation of organic water pollutants due to narrow bandgap i.e. 2.5-2.8 [18]. Recombination chances of electron-hole pairs can also occur during the excitation of an electron from the conduction band (CB) to the valence band (VB) which can slow down or stop the photocatalytic reaction. Addition of GO with Bi₂MoO₆ provides an effective charge transfer and causes a reduction in the recombination of electron-hole pairs. The conduction band (CB) of Bi₂MoO₆/GO is composed of Bi, 6p, and Mo 4p molecular orbitals whereas O 2p orbitals made the valence band (VB). GO

behave as an electron promoter by minimizing the back transfer of excited electrons and facilitates a continuous oxidation process [19].

During the photocatalysis process semiconductor $\text{Bi}_2\text{MoO}_6/\text{GO}$ photocatalyst absorbs photons of energy equivalent to its band gap energy. Absorption of photons leads to the excitation of electrons from the valence band to conduction band of the catalyst by leaving a hole in the valence band. Graphene oxide captures the excited electrons from the conduction band of Bi_2MoO_6 preventing the recombination of generated electron-hole pairs, leading to the formation of superoxide ($\text{O}_2^{\cdot-}$) and hydroxy radicals. The holes in the valence band lead to the formation of hydroxyl ($\cdot\text{OH}$) radicals. Superoxide and hydroxyl radicals behave as strong oxidizing and reducing agents to decompose the complex dye molecules into nontoxic constituents [17, 20] as shown in Figure 3.

3.3.1 Effect of Contact Time

To evaluate the effect of time for maximum degradation of direct violet 51 (DV) dye, absorption of dye solution was evaluated after regular time

intervals starting from 0-80 minutes without using any oxidizing agent to accelerate the degradation process. The characteristic absorbance at 586 nm of DV in an aqueous solution decreases more quickly under the degradation activity of the photocatalyst. Figure 4(A) shows the decrease in the absorbance of respective wavelength ($\lambda_{\text{max}} = 586 \text{ nm}$) for DV dye (20 ppm) aqueous solution (50 mL) with 50 mg of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes as photocatalyst. Figure 4 (B) shows the percent degradation of DV dye solution as a function of contact time. Results indicate that there is around 19.22 % elimination of dye was due to adsorption even at the time of adsorption-desorption equilibrium in dark. After the interaction with sunlight dye color starts to become fade showing the degradation of dye molecules. There is a gradual increase in the degradation rate up to 50 minutes which results in 97.94 % dye degradation and then the degradation rate became slow down and give 99.00 % degradation within the test period up to 80 minutes.

3.3.2 Effect of pH

pH of aqueous solution also affects the photocatalytic activity of catalysts. pH affects the surface charge

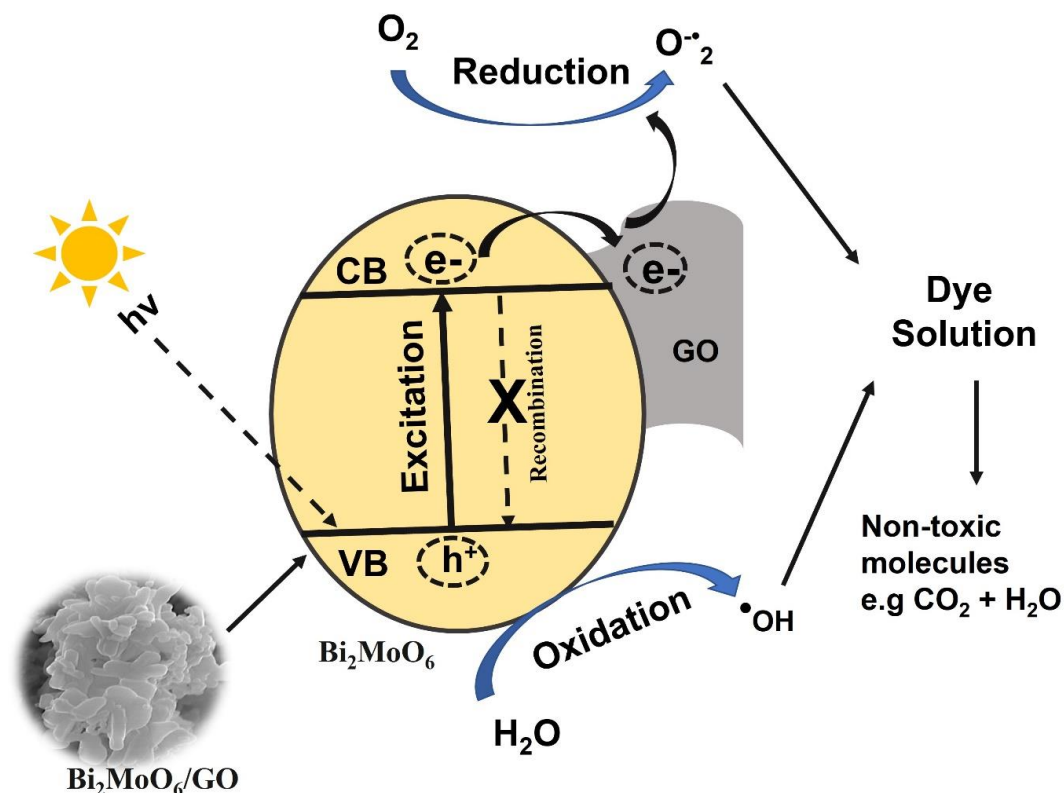


Fig. 3. Photocatalysis mechanism of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes for photodegradation of direct violet 51 dye under solar energy.

of catalysts according to their point of zero charges (pHPZC) and results in to change in the adsorption capacity of catalysts [21]. To see the pH effect of aqueous DV dye solutions on the photocatalytic efficiency of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes, the pH of dye solutions was adjusted by using 0.1 M NaOH and 0.1 M HNO_3 from 3-9. Other experimental conditions were the same, e.g dye concentration (20 ppm), volume (20 mL), catalyst dose (20 mg), and time was 60 minutes. An increase in pH from 3-7 exhibits increase in the degradation rate of dye molecules (76.14-98.70 %) and then a decrease in % degradation at pH 9 (86.82 %). Results indicate that maximum dye degradation of about 98.70 % was observed at neutral pH=7 rather than acidic and basic pH (Figures 4C and D). This phenomenon may be because at acidic pH more hydronium ions surrounds negatively charged catalyst and reduce the interactions of anionic DV dye molecules leading to a slow-down dye degradation rate. By increasing the pH value, the number of hydronium ions decreases, and the interaction of dye molecules increases towards $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes

photocatalyst leading to a higher dye degradation rate by the rapid formation of hydroxyl radicals. Further increase in pH =9, has a negative effect on the degradation process. This may be due to the fact that at basic pH more number of hydroxyl groups of basic dye solution suppresses the oxidation of water and dye molecules to generate the hydroxyl radicals. In an alkaline medium, both negatively charged catalyst and anionic dye molecules may repel each other and cause a decline in the degradation process [22].

3.3.3 Effect of Catalyst Dose

The amount of catalyst is also considered a function of the degradation process. To observe the effect of catalyst dose, dye solutions having the same concentration (20 mg/L) with the same volume (20 mL) were treated with different amounts of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes i-e 5, 25, 50, 100, and 150 mg for 60 minutes. Results have shown an increase in the rate of degradation i-e 51 to 99.99 %, with the increase in catalyst dose from 5 to 100 mg of

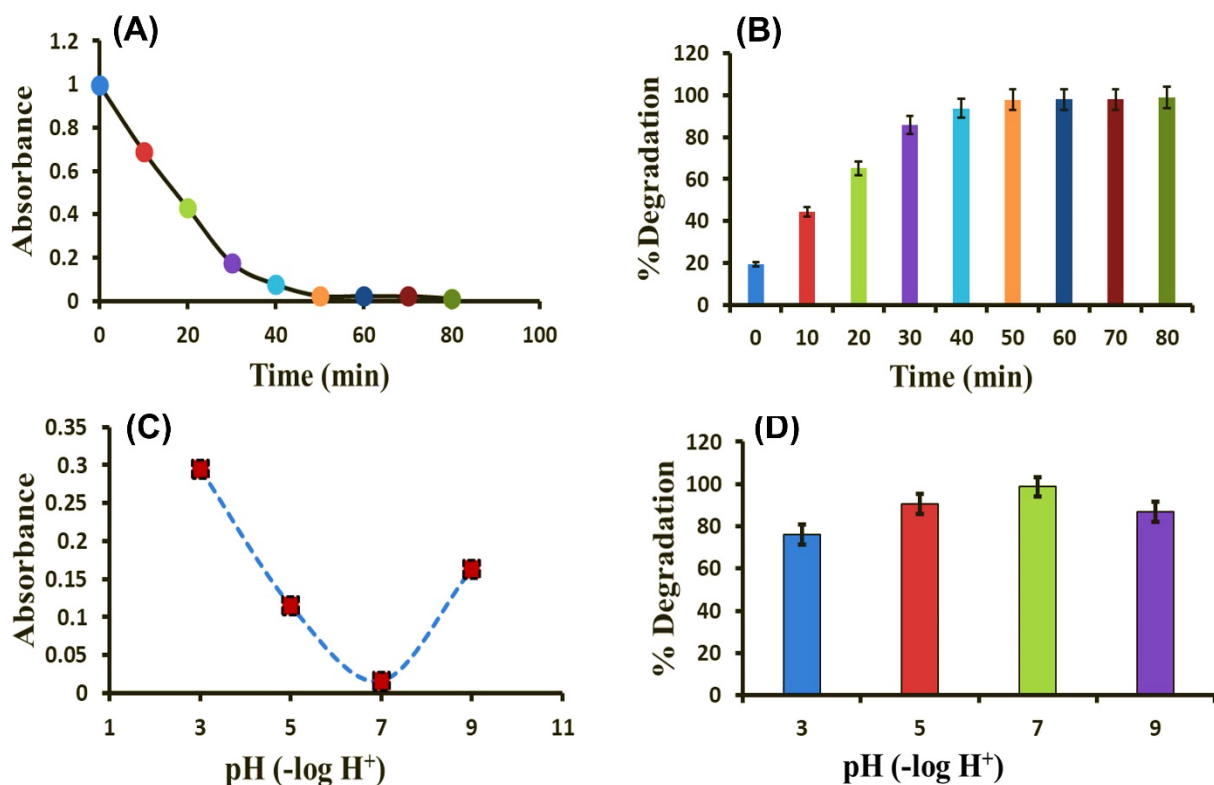


Fig. 4. Photocatalytic degradation rate of DV dye under sunlight at different time intervals with $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes as the effect of contact time for experimental parameters (A), respective % degradation of DV dye at different time intervals (B), the effect of pH on degradation efficiency of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes against DV dye (C), respective % degradation efficiency of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes at different pH values of dye solution (D).

$\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes (Figure 5A). Further increase in the amount of catalyst about 150 mg causes an 11 % decrease in the photocatalytic activity of the catalyst. It indicates that up to a certain limit increase in catalyst dose results in the production of more reactive oxidizing species to oxidize a greater number of dye molecules in less time. In this connection, beyond the optimum catalyst dose, it has a negative effect on the degradation rate. This may be due to the agglomeration of a high dose of catalyst leading to a reduction of active surface area, or it may decrease the penetration rate of light photons to the inner layers of the dye solution. Light scattering may also occur at the surface of upper-layered $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes preventing the appropriate interaction of high energy light intensity to the lower-layered catalyst surfaces [23].

3.3.4 Stability/ Reusability

In addition, to confirm the stability and reusability of the high photocatalytic performance of the $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes, recycling experiments for the photodegradation of DV were conducted. For this 20 ppm, 20 mL dye solution was treated with 20 mg of catalyst for 80 minutes. The catalyst was used again and again for seven days after washing and drying to degrade the respective new 20 mL, 20 mg/L of dye solution. Figure 5 (B) exhibits 99.82 % degradation for the 1st cycle and 93.84 % dye degradation for the 7th cycle. This 5.98 % decrease in the degradation may be due to the loss of photocatalyst during centrifugation, washing, and drying procedures. It was observed

that the same catalyst can be used again and again for the degradation of organic water pollutants. Results exhibit the good stability and reusability of the used catalyst making it cost-effective without the production of side products.

3.3.5 Kinetic Modeling

Pseudo first-order kinetic model defines such type of system where the rate of degradation depends upon the extent of the molecules of dye in the solution. The linear expression for this model can be represented as.

$$\ln\left(\frac{C_0}{C_t}\right) = k_1 t$$

C_0 is the initial concentration of DV dye at the time $(t)=0$, C_t denotes the final concentration of DV after the test period $(t)=t$, k_1 is the rate constant, and “ t ” is the time of reaction. The value of the rate constant can be calculated from the slope of the plot $\ln(C_0/C_t)$ vs time (t) . Figure 6 (A) shows the plot between $\ln(C_0/C_t)$ and time (t) for DV dye when treated with $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes to see the effect of contact time. The value of the coefficient of determination, $R^2 = 0.954$ which is near to unity indicates the best fitting of the pseudo 1st-order kinetic model on the data. Value of rate constant $k = 0.0587/\text{min}$. Similarly, Figure 6 (B) shows the plot between $1/C_t$ and time (t) to see the photocatalytic activity of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes for photodegradation of CV dye as a function of time to evaluate the effect of contact time. The

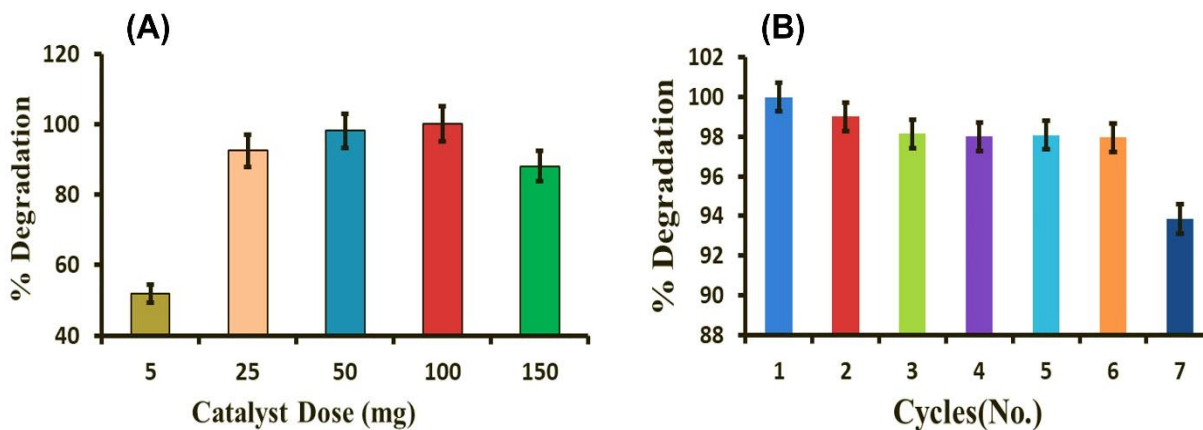


Fig. 5. % degradation of DV dye with $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes for experimental parameters i.e effect of catalyst dose (A), and reusability/stability (B).

value of the coefficient of correlation shows that $R^2 = 0.84$ which is less than 0.95 (in the case of pseudo 1st order) indicating that best fitting of pseudo first order kinetic model on the dye degradation rate of reaction. Where the value of rate constant was found to be $k = 0.0587/\text{min}$ for pseudo 1st order and 0.0573 in case of pseudo 2nd-order kinetic models. The expression for the pseudo 2nd-order kinetic model is shown as

$$\frac{1}{C_t} - \frac{1}{C_0} = k_2 t$$

3.4 Chemical Oxygen Demand (COD) for Industrial wastewater treated by $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes

Chemical Oxygen Demand (COD) was evaluated for industrial wastewater to check the efficacy of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes to purify industrial wastewater. For this, 20 mL of 20 times diluted industrial wastewater was treated with 20 mg of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes for 60 minutes under solar irradiation.

To see the photodegradation effect of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes against organic molecules in the sample wastewater before and after treatment COD was calculated. Initial COD value for untreated wastewater was found to be 83.85 mg/L, after treatment with a catalyst it was reduced to 25.8 mg/L.

$$\% \text{ COD removal} = \frac{(83.85 - 25.8)}{83.85} \times 100 = 69.23 \%$$

Percentage removal of COD after treatment

with $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes = 69.23 %

It was found that the overall chemical oxygen demand (COD) of industrial wastewater was decreased up to 69.23 % by treatment with $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes. This is due to the fact that the organic matter/ dye was oxidized in the wastewater. It was concluded that $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes have good photocatalytic potential and behave as an oxidizing agent for organic species, including dyes, which are present in the industrial wastewater. Nanostructures can oxidize harmful organic species in wastewater and convert them into nontoxic molecules e.g CO_2 and H_2O .

4. CONCLUSION

This study concluded that $\text{Bi}_2\text{MoO}_6/\text{GO}$ composite was synthesized by facile hydrothermal method with dispersed graphene oxide nanosheets. The SEM analysis revealed the sheet-like morphology of GO and flake-like for $\text{Bi}_2\text{MoO}_6/\text{GO}$ composite material. They developed an interfacial interaction during composite formation. This interaction plays a major role to suppress the recombination of electron-hole pairs by promoting the transfer of excited electrons to reduce the adsorbed oxygen on the catalyst surface. Separation of charge carriers prolongs the photogenerated charge carriers leading to the formation of more reactive oxygen species to oxidize the dye molecules. Results showed that flakes like $\text{Bi}_2\text{MoO}_6/\text{GO}$ composite give 99.00 % degradation of DV dye and reduce the 69.23 % reduction of COD for industrial wastewater under the influence of solar light. Results revealed that such nanostructures can be utilized for the

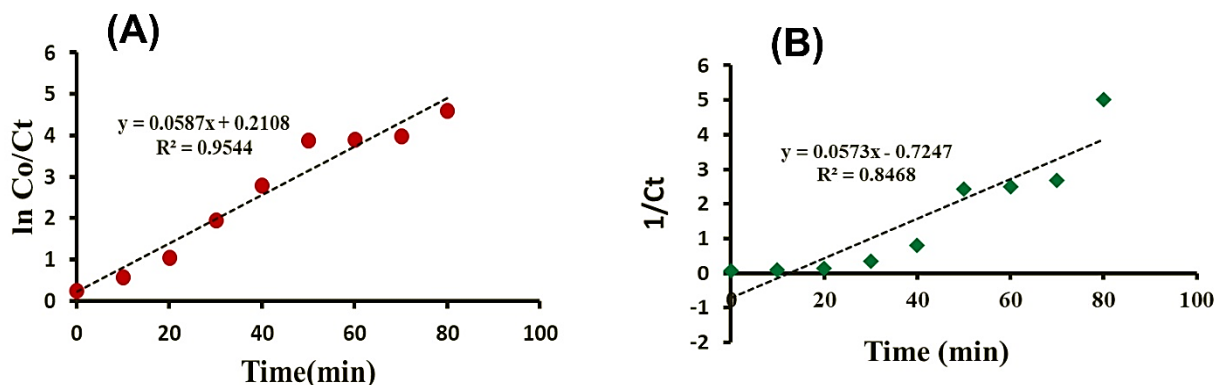


Fig. 6. Pseudo 1st order kinetic modeling, plot between $\ln(C_0/C_t)$ and time (t) for photodegradation of DV dye by the photocatalytic activity of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes (A) and plot between $1/C_t$ and time (t) for photodegradation of DV dye by the photocatalytic activity of $\text{Bi}_2\text{MoO}_6/\text{GO}$ nanoflakes (B).

degradation of organic water pollutants on an industrial scale before the discharge of wastewater into water bodies i.e. lakes, rivers, etc. These dyes are harmful to the living species and also to the environment and should be treated before their discharge into the water bodies.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

There is no conflict of interest.

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Decomplexation of Venom Proteome of Pakistani Cobra (*Naja naja naja*)

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Abstract: The venom proteome of *Naja naja* from Sindh, Pakistan was decomplexed utilizing reverse phase HPLC and SDS PAGE. The results were compared with already reported *Naja naja* species of the region. The banding pattern represents all the major families of proteins including three-finger toxins, phospholipase A₂, snake venom metalloproteases, L-amino acid oxidases, phosphodiesterase, nucleotidases, cysteine-rich secretory proteins, serine proteases, nerve growth factor, cobra venom factor, acetylcholinesterases, Kunitz-type serine protease inhibitors and C-type lectin proteins. The decomplexation of the venom showed the best possible separation through RP-HPLC elution of venom components containing small peptides, small and large proteins based on hydrophobicity. The SDS PAGE under reducing and non-reducing conditions of HPLC fractions highlighted the presence of several proteins.

Keywords: *Naja naja*, Venom, Decomplexation, HPLC, SDS PAGE

1. INTRODUCTION

Snake venomics is a comparatively new field with tools and techniques related to understanding the mechanism of venom production, its composition and pathogenesis, and also because of its therapeutic value. Decomplexation of venom proteome is particularly important as venom is a mixture of numerous proteins and peptides or toxins with multiple functionalities [1].

Cobra (*Naja naja*) is one of the deadliest species of snake found in the Indian subcontinent. *Naja naja* (*N. naja*) belongs to the family Elapidae and is commonly known as Indian Cobra in this subcontinent. In Pakistan, the specie is distributed from Khyber Pakhtunkhwa, Sindh, and Punjab to a few areas of Baluchistan [2]. *N. naja* is part of the “Big four” group of snakes which include the four deadliest species of snakes responsible for high mortality and morbidity throughout the subcontinent [3]. Common symptoms followed by cobra bite are local swelling and tissue necrosis, difficulty in speaking and swallowing, paralysis,

fixed dilated pupils and death due to respiratory failure [4].

The venom of *N. naja* has a variety of biologically active molecules divided into several classes and sub-classes. Enzymes, proteins, and small peptides are particularly important as these are the potential molecules responsible for venom pathophysiology. The main families of enzymes identified in the venom of *N. naja* are phospholipases A₂ (PLA₂), Snake venom metalloproteinases (SVMP), Snake venom serine proteases (SVSP), L-amino acid oxidases (LAAO), Endonucleases (END), Phosphodiesterases (PDEs) and Acetylcholinesterase (ACE). Three-finger peptides (3FTx), Cysteine-rich secretory proteins (CRISPs), Nerve growth factor (NGF), Cobra venom factor (CVF), and natriuretic peptides are non-enzymatic proteinous molecules with toxic effects. These have been found in the venom of *Naja* specie in several studies [5, 6]. Our study aimed to explore the venom proteome of the Pakistani cobra, *N. naja* from different geographical regions of Pakistan. Here the protein

decomplexation technique including HPLC and polyacrylamide gel electrophoresis (SDS PAGE) was utilized to analyze the cobra venom collected from the province of Sindh. Separation profiles of the venom through HPLC and SDS-PAGE were compared based on respective estimated molecular masses of the protein bands and reported studies conducted on *N. naja* specie of the subcontinent. HPLC and acrylamide gel electrophoresis are the basic protein separation techniques that are most utilized and available resources in a basic protein separation facility. Our results demonstrate the complex nature of Pakistani cobra contributing towards understanding the genus *Naja* venom in South Asia specifically in the Indian subcontinent.

2. MATERIALS AND METHODS

2.1 Sample Collection

The venom was purchased from a snake charmer who collected snake specimens from different districts of the province of Sindh and brought them to the institute where snakes were milked for venom. The venom was centrifuged at 4 °C, 7,000 rpm for 30 minutes to remove any tissue material that may have come from the mouth of the snakes. The clear venom was lyophilized and kept at -20 °C until further use.

2.2 Chromatographic separation of venom

Reverse phase high-performance liquid chromatography (RP-HPLC) was utilized for protein separation of lyophilized venom. The venom sample (2 mg) was suspended in 200 uL of 0.1 % trifluoroacetic acid (TFA) water (solvent A) which is the initial buffer to start the sample run. The reverse phase column employed was a C-18 column with 250 x 10 mm column dimensions, 5 um particle size and 300 Å pore size. A linear gradient was run with 0.1 % TFA, acetonitrile (solvent B) 5 % to 70 % B in 120 minutes. Fractions were read at 280 nm and collected manually. After collection, the samples were desiccated and subjected to SDS PAGE analysis.

2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis of crude venom was performed

under reducing as well as non-reducing conditions SDS-PAGE gels of 12.5 % or 15 % were prepared using the standard protocol by Laemmli [7]. Samples were first dissolved in water and then diluted in 2x sample diluting buffer (SDB) which was modified (2 % SDS, 2 mM EDTA, 160 mM DTT, 20 % glycerol, 0.1mg/ml bromophenol blue in 20 mM tris HCl pH.6.8), and heated at 95 °C for 3 minutes. The sample diluting buffer for the loading of crude venom samples was modified as there were insolubility issues with the sample. Gels were run at constant a voltage of 65 or 70 volts for 2 hours. Gels were stained overnight using coomassie brilliant blue and de-stained with distilled water.

3. RESULTS AND DISCUSSION

3.1 Chromatographic separation of venom

The RP-HPLC program was run to fractionate the venom sample of *N. naja* and resolve the proteins and peptides into 22 fractions (Fig. 1). The fractions were eluted at different retention times with increasing concentrations of solvent B. The gels were run under reducing and non-reducing conditions of the collected fractions. The peaks collected until 25 minutes of the run did not show any bands on the gel (data not shown). It may be due to very small peptides or nucleosides [8]. However, the eluents after 30 minutes to 95 minutes of the run (peaks from 1 to 22) showed bands of proteins and peptides.

The chromatographic separation was followed with modifications as described by Tan *et al.* [1]. The HPLC program for the separation of venom proteins was scaled down from the original 180 minutes method to 120 minutes keeping the acetonitrile gradient in a similar shape with reading the eluted peaks at 280 nm. Lomonte and Calvete depicted a similar fractionation scheme of the venom [8]. Wong *et al.* have reported the decomplexation of *N. naja* venom of Pakistan and compared it with the Indian and Sri Lankan *N. naja* specie [9]. They have reported the variation in neurotoxin components which was found to be high in Pakistan *N. naja* as compared to other countries of the region. They performed decomplexation on HPLC with 180 minutes program and marked 33 peaks. The first ten peaks showed the presence of three-finger toxins or neurotoxins [9]. In our study, the HPLC run was

120 minutes and the number of peaks collected was 22 with high resolution. The collected peaks were analyzed in reducing as well as non-reducing SDS PAGE conditions. Another comparative study [10] conducted on the venom of *N. naja* and *Naja oxiana* from Pakistan utilizing mass spectrometric analysis also showed the presence of three-finger toxins (3FTx) but the percentage composition was found to be 21 % in contrast to the previous report of decomplexation where it was found to be more than 75 % [9]. This reflects the approach with which the venom analysis was performed and the region from where the snake specimen were collected. For example, the *N. naja* venom from South Punjab Pakistan showed the presence of three-finger toxins at 58 % [10] as compared to the northern side [5]. The decomplexation strategy however presents a much more detailed and comprehensive report of proteoforms [9]. Another study that compared the Indian and Sri Lankan *N. naja* applied the same decomplexation approach and identified the presence of three-finger toxins as 74 % and 80 % respectively. The specimen of *N. naja* included in this study were collected from the Rajasthan and Gujrat, regions of India. This area is close to the province of Sindh in Pakistan. The HPLC chromatogram of this study showed a different

elution pattern with 160 minutes of program and 18 peaks [10]. However, the percentage composition of 3FTx in this study is similar to the *N. naja* venom content reported from Pakistan [9]. We report here the best possible HPLC separation of *N. naja* venom from the province of Sindh, Pakistan as compared to previous studies.

3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of venom

Crude venom was subjected to SDS PAGE under reducing and non-reducing conditions. Both gels showed protein bands ranging from as high as ~120 kDa to as low as ~6.5 kDa of molecular mass. Three individual venom samples collected from three specimens were run side by side, presenting a similar pattern of bands in both conditions (Fig. 2). The gel under reducing conditions of whole venom was compared with gels reported from India and Sri Lanka under the same condition of SDS PAGE.

The SDS PAGE results of whole *N. naja* venom was compared to the results reported by Vanuopadath *et al.* 2022 [11]. They described the proteome of *N. naja* species from Kerala, India [11]. They have identified the bands obtained from

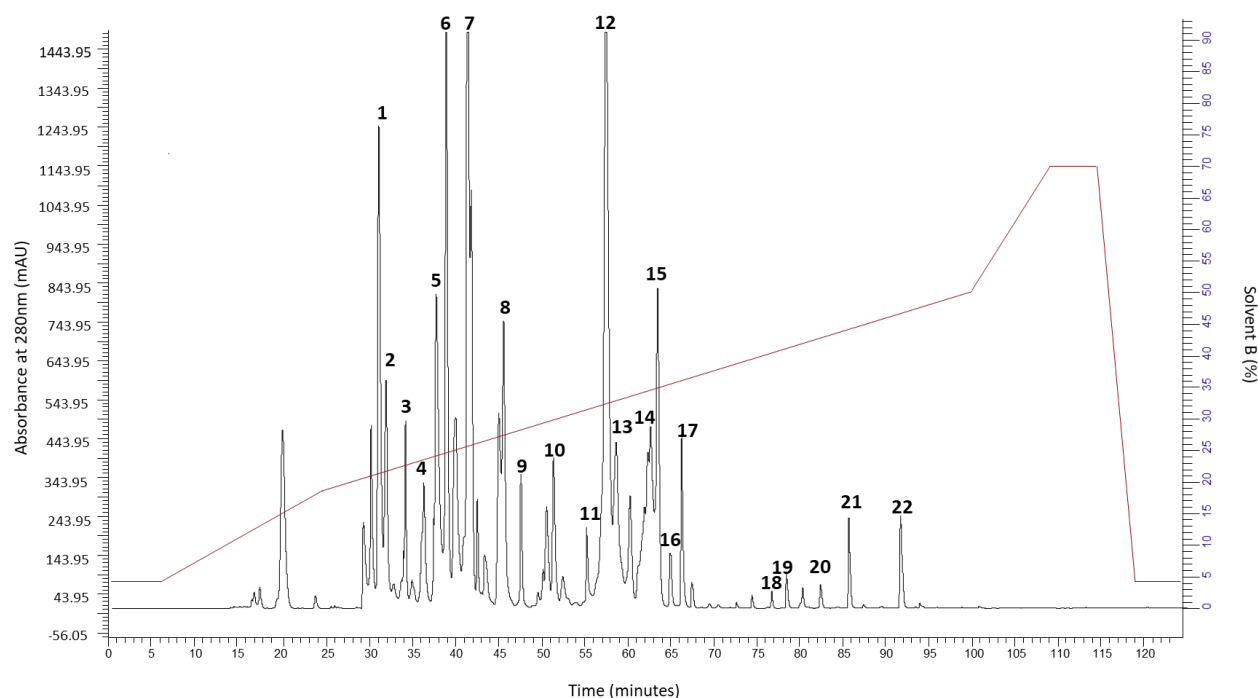


Fig. 1. HPLC chromatogram of cobra (*Naja naja*) venom on C18 column. The numbers represent the collected peaks.

similar SDS PAGE procedures performed under reducing conditions. Our gel results represent an almost similar band pattern (Fig. 3).

Based on the band comparison the expected identification of proteins could provide an idea of the groups of proteins present in each band marked in figure 3 (A). To elaborate on such a scenario, band 1 might represent snake venom metalloprotease (SVMP), cobra venom factor (CVF), and phosphodiesterases (PDE). Band 2-4 represents acetylcholinesterase (ACE), 5'-nucleotidase (5-ND), snake venom serine protease (SVSP), Phospholipase B (PLB) and L-amino acid oxidase (LAAO) in addition to SVMP and CVF. Band 5 may include endonuclease (END) in addition to SVMP, CVF and LAAO. Similarly bands 7 and 8 might comprise of Ohanin/vespryn family proteins (OLP), cysteine-rich secretory protein (CRISP), glutathione peroxidase (GPrx), phospholipase A₂ (PLA₂), C type lectin (CTL) and nerve growth factor (NGF). Band 9 symbolizes three-finger toxins (3FTx) in addition to OLP and PLA₂ whereas, band 10 could include Kunitz-type serine protease inhibitor (KSPI) with 3FTx, PLA₂, and OLP.

Fractions collected from HPLC were subjected to SDS PAGE to determine the protein band pattern. Multiple bands were found in each

collected fraction on gel under both reducing and non-reducing conditions. The banding pattern obtained under reducing SDS PAGE conditions were compared with the already reported gels of *N. naja* venom [9-11]. Fractions 3, 4 and 5 mainly showed low molecular mass proteins (~7 kDa). Chromatographic peaks from 6 to 13 presented bands in the range of molecular masses of ~85 to 6.5 kDa. The rest of the peaks showed the presence of high molecular weight proteins (~140 to 55 kDa). Over all more than 40 bands were observed in reducing SDS PAGE gels of the HPLC collected fractions. Hashmi *et al.* reported the *N. naja* venom decomplexation utilizing the 180 minutes' acetonitrile gradient program and analyzed the collected fractions on 12.5 % SDS PAGE gel [12]. In our study, we analyzed the first 9 fractions on 15 % gels and the rest of the fractions on 12.5 % SDS PAGE. We adopted the strategy based on the literature review where it was observed that the 3FTx are the main eluent in the initial phase of the HPLC program. The analysis of fractions under non-reducing conditions was performed to have an idea about the fractions with proteins consisting of more than one subunit. Fractions 6 and 10 showed the presence of such proteins (Fig. 4). Most of the literature report only reducing SDS PAGE gels of the collected venom fractions. However non-reducing SDS PAGE could provide functional

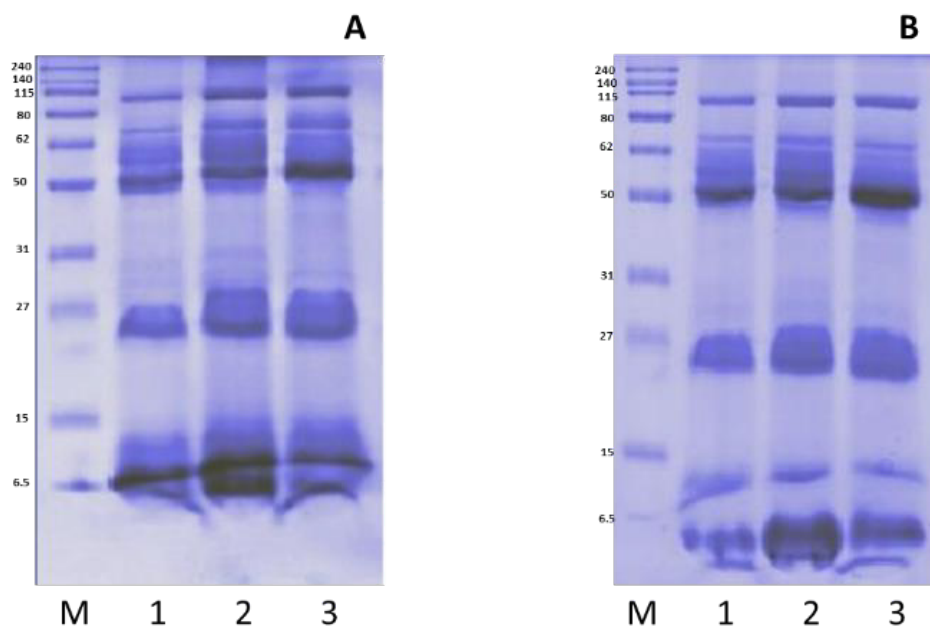


Fig. 2. 12 % SDS PAGE under non-reducing (A) and reducing (B) conditions. 'M' represents the molecular weight marker with respective bands in kDa in lane 1. From lane 2-4 the number represent venom samples of three individual specimens of *Naja naja*.

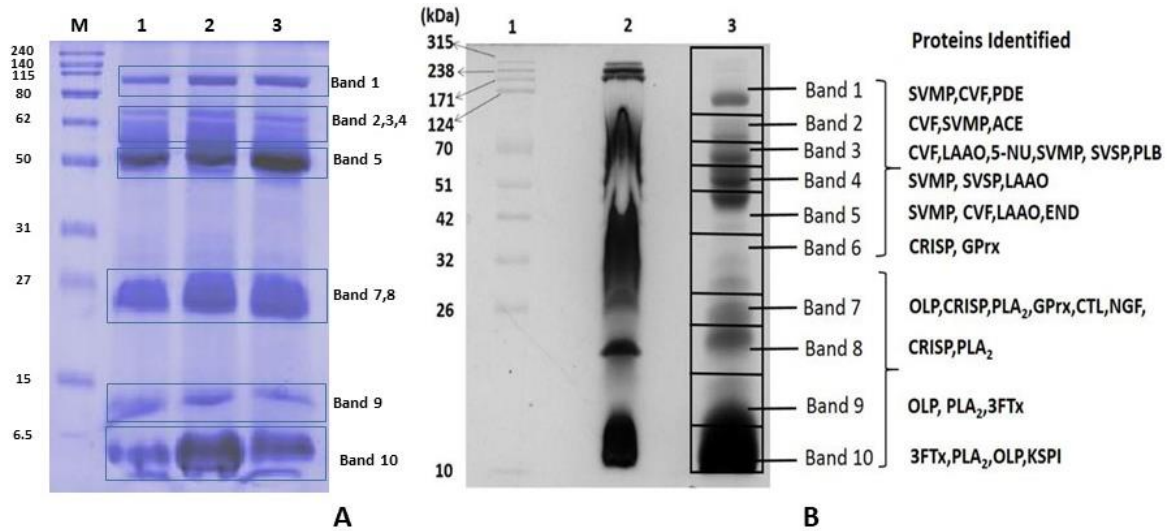


Fig. 3. 12.5 % SDS PAGE under reducing conditions (A) where bands are marked in boxes in comparison to the Vanuopadath *et. al.* 2022 [11]. ‘M’ represents the molecular weight marker with respective molecular mass (kDa) in lane 1. From lane 2-4 the number represent venom samples of three individual specimens of *Naja naja* venom. Gel image opted from Vanuopadath *et. al.* 2022 [11] (B) representing molecular weight marker in lane 1, whole venom of *N. naja* under non-reducing conditions in lane 2 and in reducing conditions in lane 3. The proteins identified in each band are mentioned on the right side of the image.

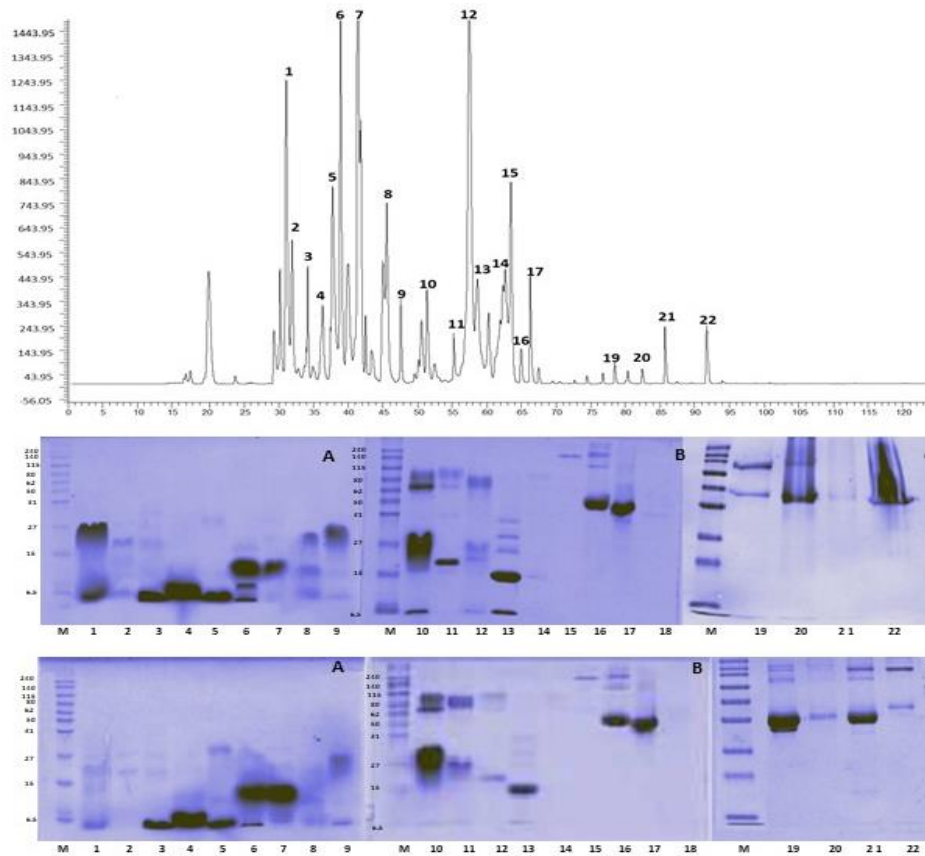


Fig. 4. Biochemical characterization of *N. naja* venom. Upper panel RP-HPLC chromatogram of the venom with collected fractions (1-22). Middle panel, reducing SDS PAGE gels and lower panel non-reducing SDS PAGE of the fractions. 15 % gel (A), and 12% gels (B and C) marked by fraction or peak numbers at the bottom. ‘M’ represents the molecular weight marker with respective molecular weights (kDa).

information as the non-denaturing conditions signify the intact interactions of proteins that are necessary for biological activities [12].

The separation pattern of the *N. naja* venom showed the generalized elution of proteins and peptides. A linear acetonitrile gradient starting from 5 % and reaching up to 50 % elutes small peptides, followed by small proteins, medium-sized proteins and large or most hydrophobic proteins in the sequence of the HPLC program [8]. The pattern of HPLC separation on reversed phase C18 column is almost similar in most studies reporting the decomplexation of cobra venom even with unique findings. For example, venom from a cobra species *Naja senegalensis* showed an absence of the Phospholipase A₂ component and high levels of 3FTx (~76 %). The specie belongs to the Western Africa subgenus *Uraeus* of genus *Naja* highlighting insights that could be used for the availability of region-specific antivenom and reducing envenomation-related deaths [13]. The decomplexation studies of snake venom target the ultimate goal of anti-venom development. The region-specific antivenom against a specific specie of snake, such as cobra should include all the members of that genus present in a particular region. This strategy would support the development of a remedy or anti-venom that would provide an effective treatment against envenomation. Our data and similar work find its contribution attention to understanding the complexity of *N. naja* venom in South Asia specifically in the Indian subcontinent.

4. CONCLUSION

The decomplexation of *Naja naja* venom presented all the major proteins in the venom reported from the *N. naja* species in the Indian subcontinent including different regions of Pakistan, North India, South India and Sri Lanka. We report the best possible HPLC separation of *Naja naja* venom from the province of Sindh, Pakistan as compared to previous studies. Fractions 6 and 10 showed the presence of proteins with more than one subunit as demonstrated by SDS PAGE results under non-reducing conditions. Our data highlights the complexity of the Pakistani cobra species *Naja naja naja*.

5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

6. DECLARATION

The author declared no conflict of interest.

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Characterization of Titanium dioxide (TiO₂) Nanoparticles Biosynthesized using *Leuconostoc* spp. Isolated from Cow's Raw Milk

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Abstract: Nanotechnology is a continually expanding field for its uses and applications in multiple areas i.e. medicine, science, and engineering. Biosynthesis is straightforward, less-toxicity, and cost-effective technology. TiO₂ NPs biosynthesis has attained consideration in recent decades. In this study, probiotic bacteria were isolated from cow's raw milk samples, and then were identified by using the Vitek2 system; as *Leuconostoc* spp. included *Leuconostoc mesenteroides* subsp. *mesenteroides* (Leu.1), *Leuconostoc mesenteroides* subsp. *cremoris* (Leu.4), and *Leuconostoc pseudomesenteroides* (Leu.14). All *Leuconostoc* spp. isolates showed an ability for TiO₂ NPs bio-production, after being incubated at anaerobic conditions (30 °C/ 24 h) in DeMan Regosa and Sharpe (MRS) broth medium. The biosynthesized TiO₂ nanoparticles were characterized using the following apparatuses: UV-Vis spectroscopy, X-ray diffraction (XRD) apparatus, Atomic Force Microscopy (AFM), Fourier Transform Infrared Spectroscopy (FTIR), Field Emission Scanning Electron Microscopy (FE-SEM) in addition to Energy Dispersive X-ray analysis (EDX) spectra. The characterized biosynthesized TiO₂ NPs were spherical-shaped, nanostructure anatase crystals with an average size range of 53.35-59.41 nm. The UV absorption spectrum was observed at the wavelength 344-248 nm; the topography AFM 2D and 3D images result showed the height and roughness of biosynthesized TiO₂ NPs that were in the range of 1.137-18.88 nm. Absorption peaks in the FTIR spectra were located in a region typical of TiO₂ NPs, and biosynthesized TiO₂ nanoparticles' main IR topographies (408.21- 445.80) cm⁻¹ belonged to anatase Titania (Ti-O-Ti) bridge.

Keywords: Biosynthesis, *Leuconostoc* spp., TiO₂ Nanoparticles

1. INTRODUCTION

Leuconostoc species are Gram-positive (G +ve), mesophilic, non-motile, non-spore forming and facultative anaerobic/ aero tolerant bacteria [1]. They are negative (-ve) for oxidase and catalase tests and they appear under the microscope as pairs and chains [2, 3]. *Leuconostoc* can be isolated from the raw milk of goats, camel, plant matter and vegetables like carrots, sauerkraut, and banana [4, 5]. They are involved in food production and industrial processes such as the production of extracellular homopolysaccharides like α glucans (dextran and alternan), and β fructans (levan and inulin) from a substrate that contains sucrose [5, 6].

There are three kinds of approaches for nanoparticle production, physical; chemical and biological methods [7]. When compared to traditional chemical and physical procedures, the biological method offers an eco-friendlier option for producing nanoparticles. In addition, biological procedures have been widely used recently, allowing for NPs to synthesize in varying forms, sizes, and contents [8]. Eco-friendly, rapid, easy, safe, energy-efficient, affordable, and less toxic, green synthesis of biogenic nanoparticles from plants or microbes or their products has become promising. Remarkably, NPs have a crucial role in tumor diagnosis in its early stages by enabling cellular visualization [9, 10].

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Extensive research has shown that probiotic bacteria can yield a wide variety of metals and oxides nanoparticles, including TiO₂, Gd₂O₃, Se, Ag, Au, CdS, ZnO, Sb₂O₃, besides many others [11,12] developed a reliable, eco-friendly, and rapid process for extracellular titanium dioxide nanoparticles based on *Lactobacillus crispatus* isolated from healthy women vagina.

In addition to their applications in photocatalytic activity and solar energy, TiO₂ NPs are attractive antimicrobial compounds for their non-toxicity, chemical stability and cost-effectiveness [13, 14]. The strong oxidizing power by free radical generation greatly improved TiO₂ NPs potential as antimicrobial candidates [14]. The current study aimed to use *Leuconostoc* spp. isolated from cow's raw milk for TiO₂ NPs biosynthesis and then confirm this result by characterization. It's an important alternate, safe, inexpensive method besides being green as eco-friendly for nanoparticle microbial (non-pathogenic bacteria) biosynthesis [13].

2. MATERIALS AND METHODS

2.1 Isolates of Bacteria

Leuconostoc spp. were isolated from cow's raw milk, and then the identification was throughout the cultural [MRS agar media was used for incubation, at anaerobic conditions (30 °C/24 h)], microscopically and biochemical test [15]; besides using Vitek2 system.

2.2 Biosynthesis of TiO₂ Nanoparticles

DeMan Regosa and Sharpe (MRS) liquid medium (40 mL) provided with 2 % (9×10⁸ CFU/ mL) of *Leuconostoc* spp. The pure culture was added to TiO₂ (20 mL, 0.025 M). Additional two formulations were prepared as mentioned above with either bacterial growth or TiO₂ eliminated to serve as the control. After stirring for 60 min, the preparations were incubated anaerobically (30 °C/ 24 h). Compared with the control, the preparation containing bacterial growth along with TiO₂ changed to dark brown sediment, indicating an initial production of TiO₂ NPs [16]. The product was purified by repeated centrifugation (5000 rpm/5 min.) and washing with deionized water. The pellet was dried at 50 °C for 1 h [17].

2.3 Biosynthesized TiO₂ NPs Characterization

Biosynthesized TiO₂ NPs were characterized via the following apparatus: UV-Vis at Ibn Sina Research Center (Ministry of Industry and Minerals/ Iraq); while XRD apparatus, AFM, FE-SEM besides EDX spectra were done by sending all samples as a powder to Research Center in (Tehran/ Iran). In addition, FTIR was measured at Mustansiriyah University/ College of Science/ Physics Department.

3. RESULTS AND DISCUSSION

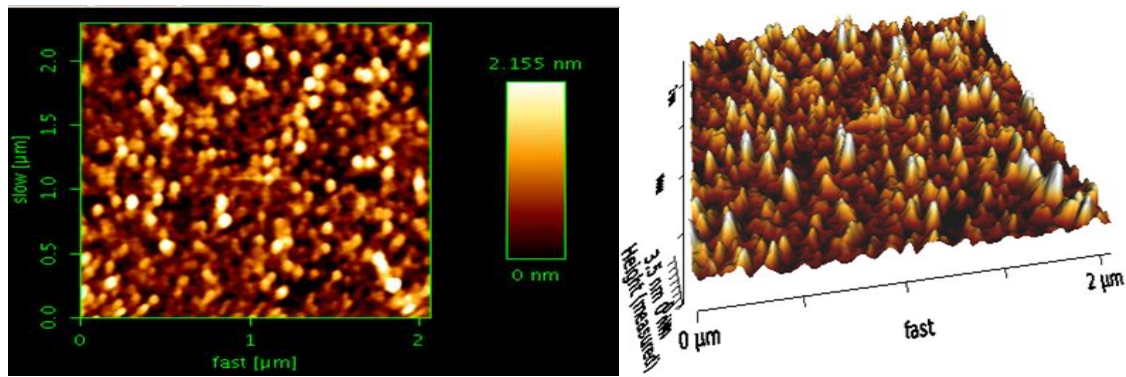
3.1 Identification of Probiotics Bacteria

Thirteen isolates, *Leuconostoc mesenteroides* subsp. *cremoris* (8), *Leuconostoc mesenteroides* subsp. *mesenteroides* (3), and *Leuconostoc pseudomesenteroides* (2) were identified using the Vitek2 system (its probability identification ratio ranged from 98-95 %, 97-90 % and 88, 86 % for all 13 *Leuconostoc* spp. respectively).

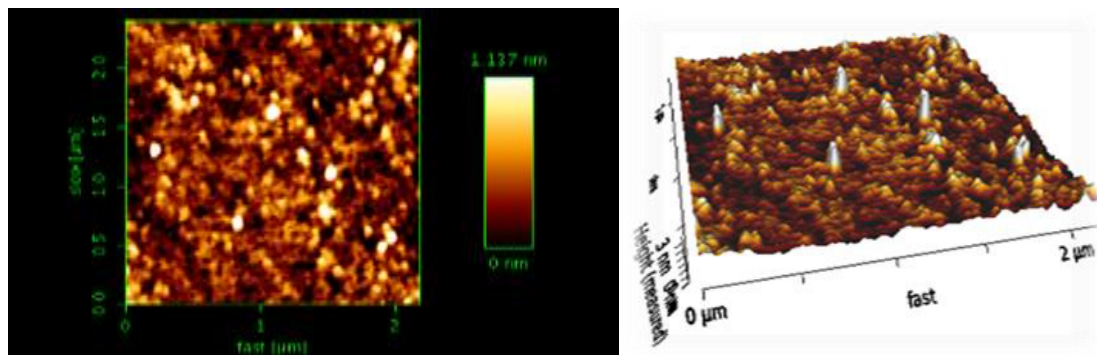
3.2 Characterization of TiO₂ NPs

The results showed that *Leuconostoc* spp. have been capable of biosynthesizing TiO₂ NPs intracellularly. UV absorption spectrum observed at the wavelength 344-348 nm for all 3 was the same as those obtained with previous TiO₂ nanoparticles synthesized by probiotics isolates, demonstrating anatase TiO₂ NPs formation [18]. The UV spectra reported by Landage *et al.* (2020) indicated a distinctive absorption peak approving the anatase phase of TiO₂ nanoparticles that were biosynthesized by *Staphylococcus aureus* and it was detected at 324 nm [19].

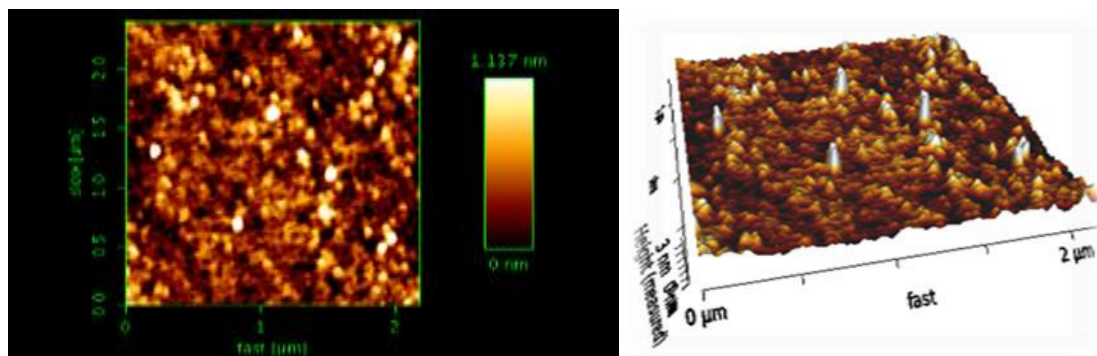
The topography AFM images with 2D and 3D images were demonstrated in Figure (1A, 1B, 1C) for biosynthesized TiO₂ nanoparticles from selected *Leuconostoc* spp. (*L. mesenteroides* subsp. *mesenteroides* (Leu.1), *L. mesenteroides* subsp. *cremoris* (Leu.4), *L. pseudomesenteroides* (Leu.14) as 2.155, 1.137, and 18.88 nm respectively. AFM notably depicted the formation of anatase formulas in TiO₂ NPs, besides particles surface morphology that was owing to the occurrence of some aggregates and individual particles. No linear trend was seen



A. *L. mesenteroides* subsp. *mesenteroides* (Leu.1), the height of the particle was 2.155 nm.



B. *L. mesenteroides* subsp. *cremoris* (Leu.4), the height of the particle was 1.137 nm.



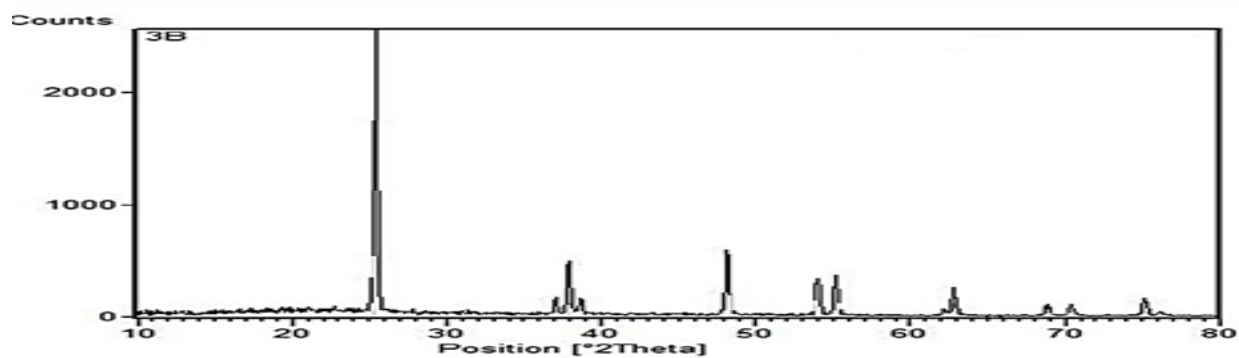
C. *L. pseudomesenteroides* (Leu.14), the height of the particle was 18.88 nm.

Fig. 1. AFM of TiO₂ nanoparticles biosynthesized by *Leuconostoc* spp. the highest of TiO₂ surface asperities were belong to (Leu.14), while the lowest was related to (Leu.4).

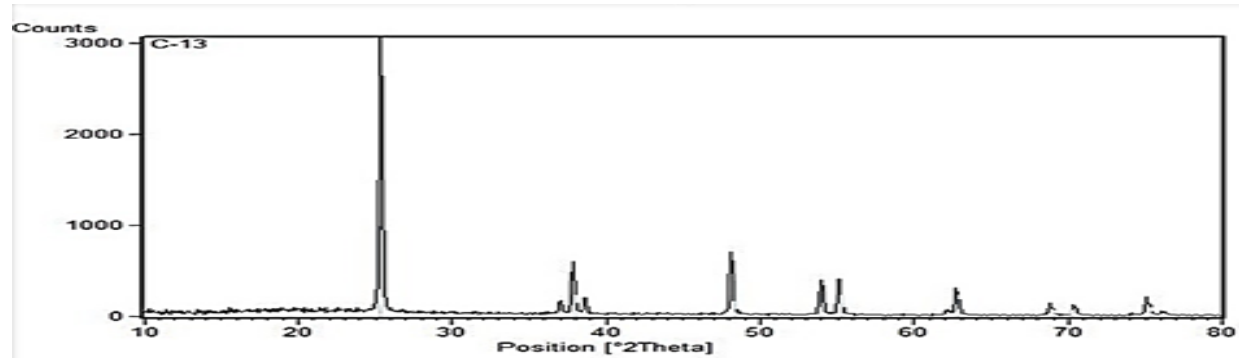
in roughness; however, that proved the highest of TiO₂ surface asperities lead to the materialization of smoother layers [19].

Identification of minerals based on their diffraction pattern is one of the main applications of XRD research. In addition to phase determination, XRD provides information on how interior stresses and defects cause the real structure to diverge from the ideal one [20]. Results of biosynthesized TiO₂ nanoparticles were analyzed by XRD; TiO₂ peaks

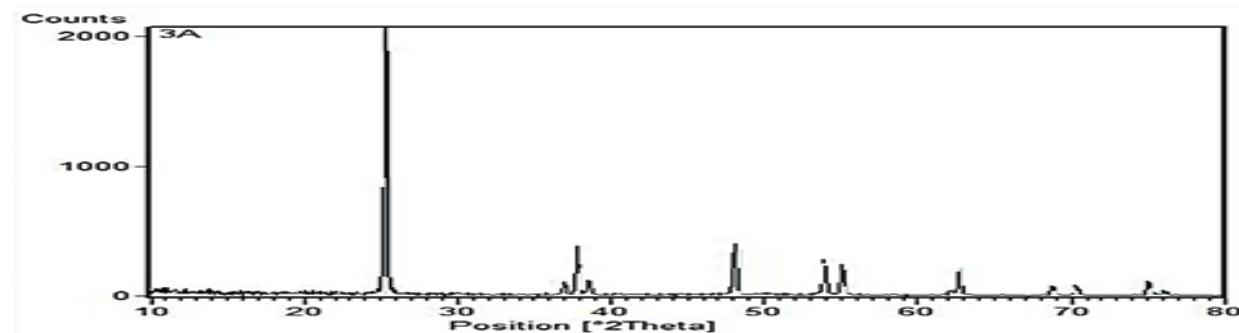
were detected at (25.2°), corresponding to 101, characteristic line broadening of diffraction peak was because of the nano size nature of anatase crystals (Figure 2). $2^{\theta}=25.2$, matches (101) the anatase crystallographic plane of TiO₂ NPs, demonstrating that the structure of nanoparticles agrees with anatase crystalline which was regarded as an indicator of TiO₂ NPs synthesized biologically [20]. Between TiO₂ crystalline phases, the anatase phase was defined as the greatest active photocatalytic one [21]. Additionally, TiO₂ layers



A. *L. mesenteroides* subsp. *mesenteroides* (Leu.1).



B. *L. mesenteroides* subsp. *cremoris* (Leu.4).



C. *L. pseudomesenteroides* (Leu.14).

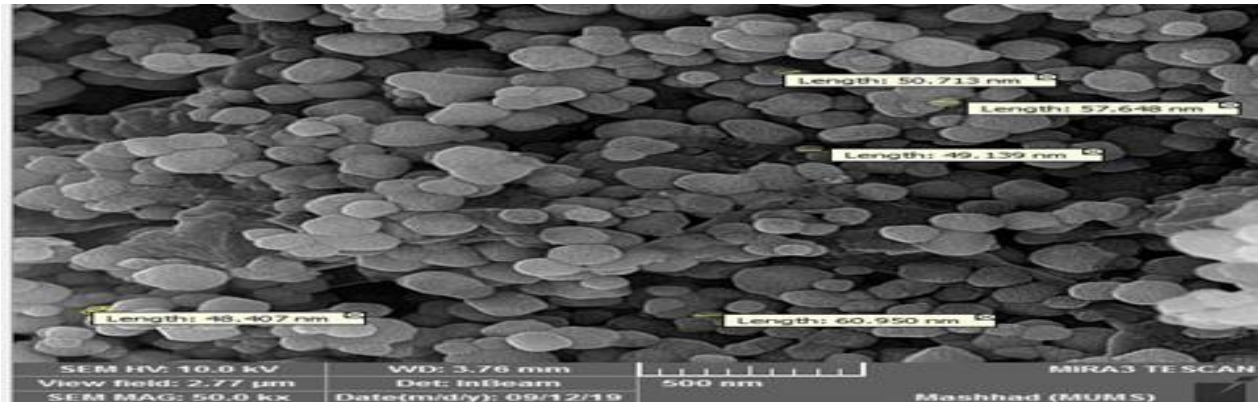
Fig. 2. XRD pattern of TiO₂ nanoparticles biosynthesized by *Leuconostoc* spp.

in the anatase phase typically work as a super hydrophilic surface [22]. High crystalline nature of TiO₂ NPs, which was indicated by the sharp peaks, favored photocatalytic activity [23]. Due to anatase's highest photocatalytic activity, has the most uses in industry and is the most widely utilized commercially [24].

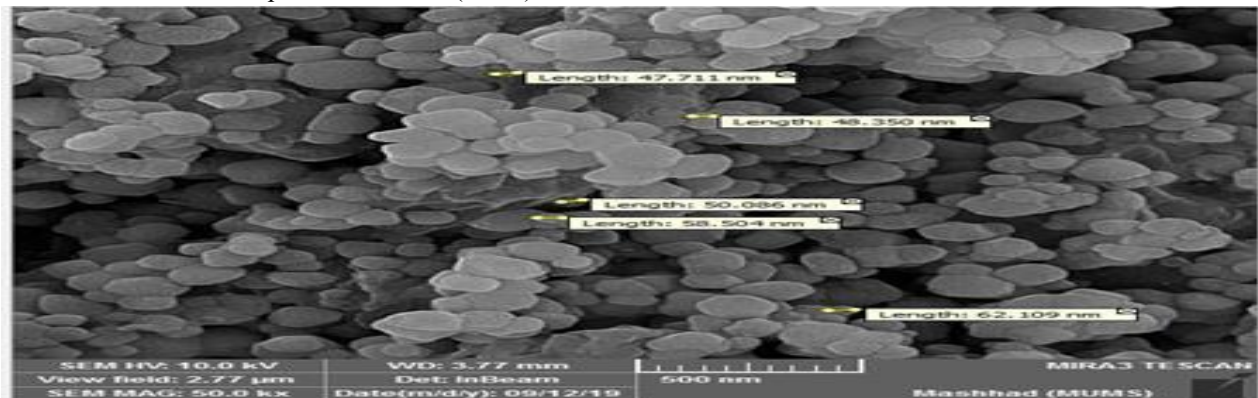
The surface micrograph of titanium dioxide nanoparticles synthesized by *L. mesenteroides* subsp. *mesenteroides* (Leu.1), *L. mesenteroides* subsp. *cremoris* (Leu.4), *L. pseudomesenteroides*

(Leu.14), consisted of a uniform distribution of spherical-shaped nanostructure crystals with an average diameter of each isolate (53.37, 53.35, 59.41) nm respectively (Figure 3).

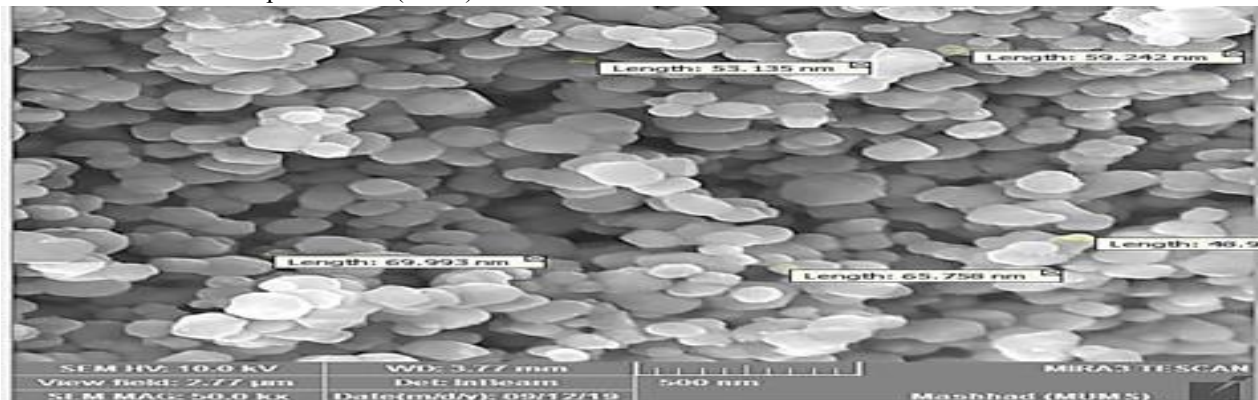
Field Emission Scanning Electron Microscopy identified the physical morphology and size of biosynthesized TiO₂ NPs. The particle size and shape, besides surface morphology that was studied by the SEM, were different. This might be related to different bacteria used in the synthesis process or because the TiO₂ nanoparticles were



A. *L. mesenteroides* subsp. *mesenteroides* (Leu.1).



B. *L. mesenteroides* subsp. *cremoris* (Leu.4).



C. *L. pseudomesenteroides* (Leu.14)

Fig. 3. FES-EM of TiO₂ nanoparticles biosynthesized by *Leuconostoc* spp.

being formed at different times [25]. According to the FE-SEM image, sphere-like particles have different diameters of 70–90 nm were observed for the biosynthesized TiO₂ nanoparticles, which might be related to particle aggregation phenomena [26]. Generally, the decrease in particle size is inversely proportional to the surface volume of the material. Consequently, the smaller the particle size, the quicker enters the toxic components along with the bacterial surface which drove decomposition [27].

exhibited that, TiO₂ NPs powders prepared from *L. mesenteroides* subsp. *mesenteroides* (Leu.1), *L. mesenteroides* subsp. *cremoris* (Leu.4), *L. pseudomesenteroides* (Leu.14) contained (Ti and O), confirming the purity of biosynthesized TiO₂ nanoparticles (Figure 4).

The EDX result of TiO₂ demonstrated that particles were in crystalline nature plus indeed in metallic TiO₂ nanoparticles [28].

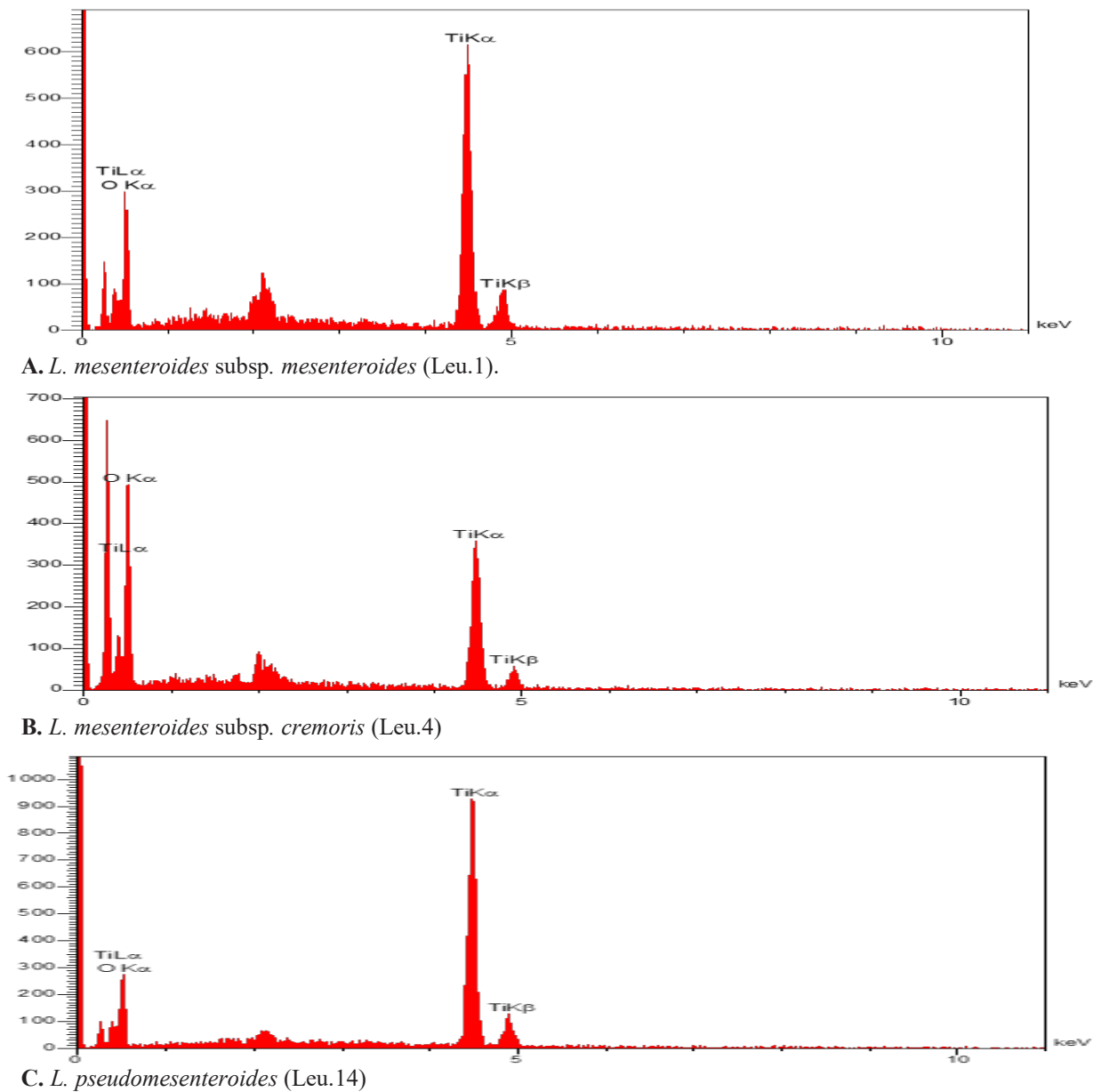
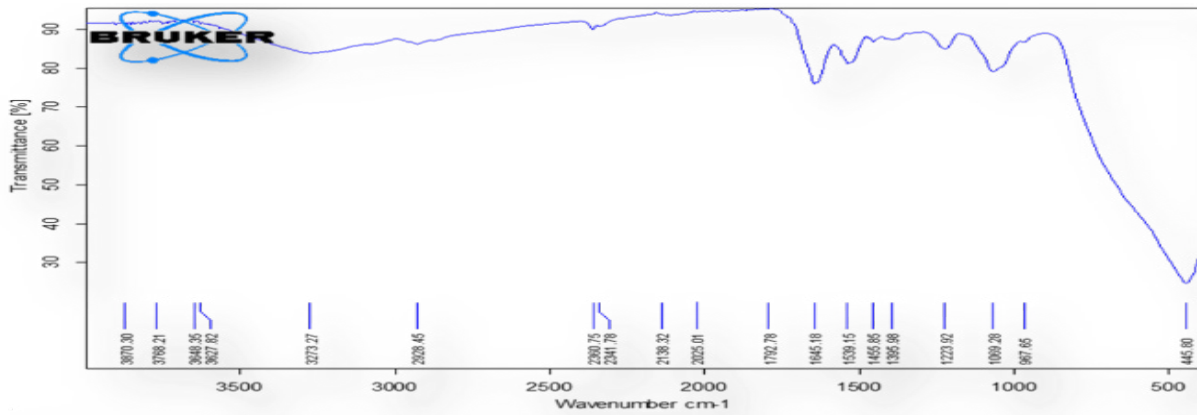


Fig. 4. EDX result of TiO₂ nanoparticles biosynthesized by *Leuconostoc* spp.

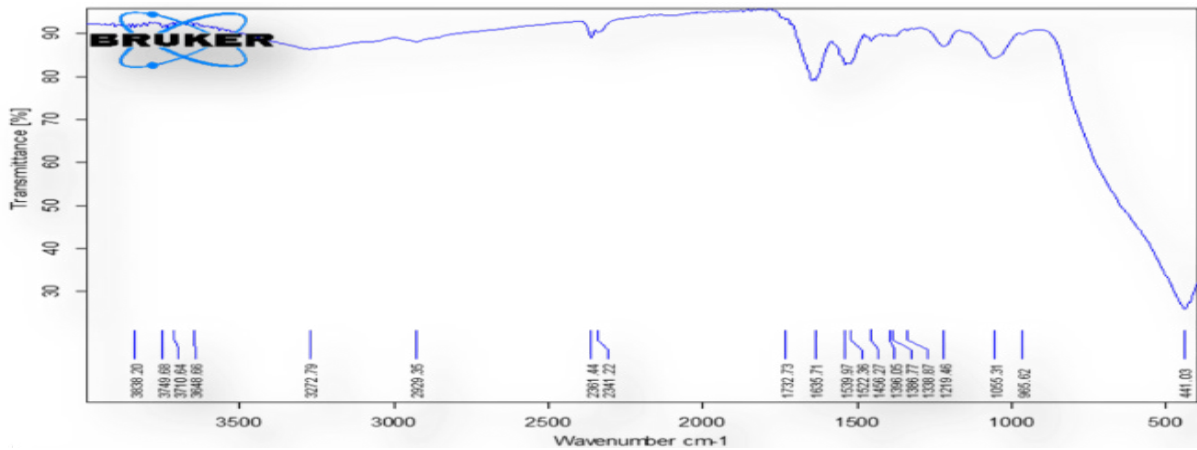
used for vibrational information of biosynthesized TiO₂ nanoparticles. It is responsible for information on the fingerprint regions of the chemical bonds within molecules. Figure (5A, 5B, 5C), demonstrated that an absorption peak was within the range spectrum that was related to TiO₂ NPs, and main IR topographies of TiO₂ nanoparticles biosynthesized by probiotics bacteria *L. mesenteroides* subsp. *mesenteroides* (Leu.1), *L. mesenteroides* subsp. *cremoris* (Leu.4), *L. pseudomesenteroides* (Leu.14) respectively, were: first peak at (445.80, 441.03, 408.21) cm⁻¹ respectively for *Leuconostoc* isolates mentioned above; these peaks were belonging

to anatase Titania (Ti-O-Ti) bridge or called (Ti-O) stretching bond, as reported in the study of Praveen *et al.* (2014), Dodoo-Arhin *et al.* (2018), they mentioned the range of anatase titanium wavenumber as (400- 1000) cm⁻¹ [29, 30].

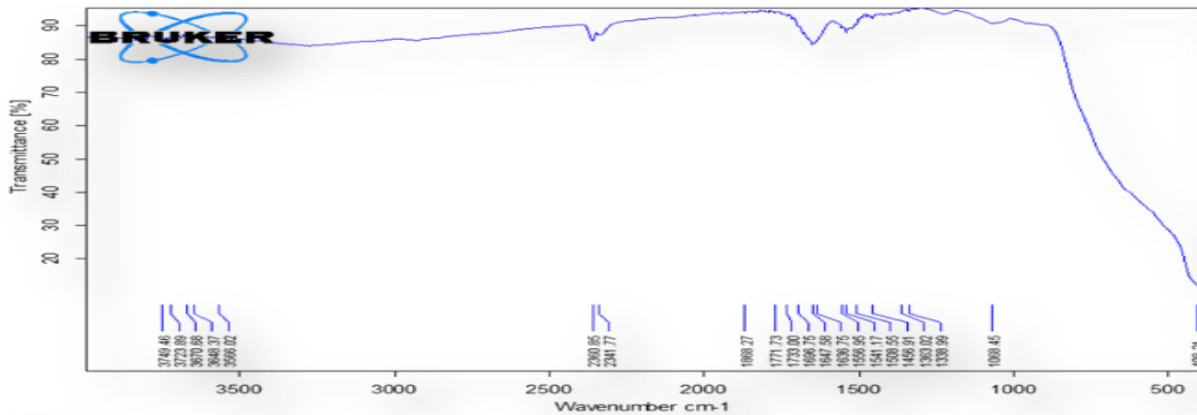
Peaks at (3273.27, 3272.79, 3273) in the 3600-3050 cm⁻¹ region might relate to the O-H stretching mode of surface and adsorbed water molecules (Figure 5). The peaks at (2360.45, 2361.44, 2360.85) cm⁻¹ for isolates in the same order. These peaks are assigned to the symmetric stretch (C-H) of CH₂ and CH₃ groups of aliphatic chains [31]; or



A. *L. mesenteroides* subsp. *mesenteroides* (Leu.1).



B. *L. mesenteroides* subsp. *cremoris* (Leu.4).



C. *Leuconostoc pseudomesenteroides* (Leu.14).

Fig. 5. FTIR result of TiO₂ nanoparticles biosynthesized by *Leuconostoc* spp.

might be related to hydrogens vibration from the hydroxyl layer [32].

The OH stretching band contributed either by molecularly adsorbed water or by surface OH.

In addition to reducing particle aggregation, and retaining their photoactivity in high concentrations. High surface OH groups of TiO₂ nanoparticles can also obstruct electron-hole recombination. The increased bridging hydroxyls on the surface

decrease the positive charge of TiO₂ and led to particle aggregation, negatively affecting catalytic activity [33].

Multiple studies have shown that increasing the number of surface hydroxyl groups on the TiO₂ surface enhances the material's adsorption capacity, mesoporous structure formation, and catalytic efficiency [34, 35].

4. CONCLUSION

The current study was focused on a quick and environmentally friendly process of creating TiO₂ NPs using *Leuconostoc* spp. isolated from cow's raw milk, the biosynthesized and characterized pure anatase TiO₂ nanoparticles within average size ranged from (53.35- 59.41) nm.

5. ACKNOWLEDGMENTS

Thanks to Mustansiriyah University-Iraq (www.uomustansiriyah.edu.iq) for supporting the current study.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest

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8. DECLARATION

The authors declared that: (i) the results are original; (ii) the same material is neither published nor under consideration elsewhere; (iii) approval from all authors

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