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Review Article

Striking Applications of Keratinase Enzyme Isolated from Various Natural Sources: A Review

Ikram ul Haq[1,2*], and Fatima Akram[1]

[1]Institute of Industrial Biotechnology, GC University, Lahore
[2]Pakistan Academy of Sciences, Islamabad

Abstract: Keratinase enzymes are protein hydrolyzing enzymes belonging to the serine hydrolase group and act specifically on keratin proteins to degrade them. Since keratin proteins are present in hair, hoofs, nails, beaks, animal skins, feathers and most of our green wastes, leaving them untreated may lead to environmental pollution. So, treating such material with keratinase enzyme in order to reduce environmental pollution is one of the striking applications of keratinase enzymes. Other applications may include dehairing, use in cosmetics, drugs, clothing and biofuel production. In this study, major focus has been given to applications of keratinases; however, the methods and sources of isolation of keratinase enzymes from different microbial sources has also been discussed. Bacteria as well as fungi possess the ability to produce extracellular keratinases which may be isolated and applied to several industrial sectors. Substitution of chemical agents with keratinase has been emphasized because in comparison to chemicals, keratinase enzymes are eco-friendly, biodegradable, do not produce harmful by-products and give very efficient results.

Keywords: Dehairing, Keratin, Keratinolytic enzyme, Serine, Domains, *Bacillus.*

1. INTRODUCTION

Keratins are the proteins which are usually present in two forms, namely hard keratins and soft keratins. Hard keratins mainly include the structural proteins which are prevalently present in finger nails, horns, beaks, upper layer of skin and mainly hair [1] (Fig. 1). Fibres of the keratin proteins are self-assembled into compact follicles that make up the structure of hair. The process of assembling up of keratin proteins into a complex hair is under the control of multiple genes, cytokines and growth factors [2]. In contrast to hard keratins, soft keratins are those which are abundantly present in tissues such as epithelial tissues.

Since the major focus of this study is on keratin proteins present in hair so we will discuss the classification of hair keratins here. The structure of wool keratin possesses great similarity with the hair keratin as shown in figure 2 [3]. Three types of hair keratin have been known yet [4]. First one is the alpha keratins, these ranges in size from 60 to 80 kDa. Being very less in sulfur content, these

Fig. 1. Sources of keratin. Different sources such as feathers, hair, nails, horns, hooves, and beak are shown. The hosts for these sources include human, bird, and animal. The hardness of these keratin materials is different in each case.

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* Corresponding Author: Ikram ul Haq; ikmhaq@yahoo.com

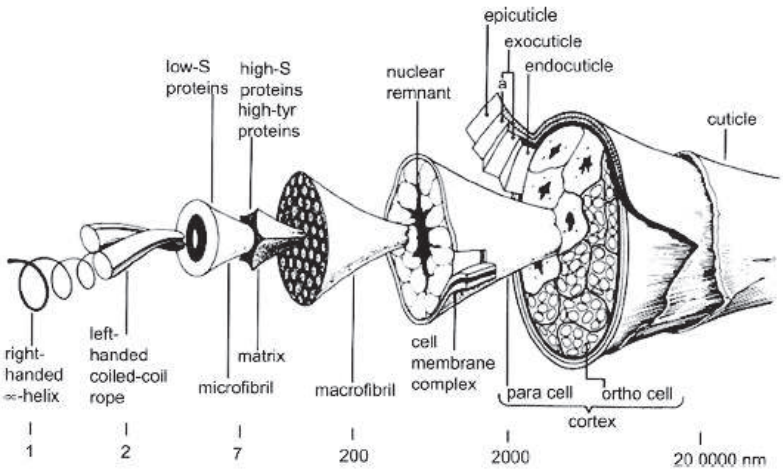

Fig. 2. The image above shows the structural scheme of keratin present in wool fibre which is closely related to human hair keratin with respect to its structure [3].

Family & Domains

Domains and Repeats

Feature key	Position(s)	Description	Actions	Graphical view	Length
Domain	19 – 77	Inhibitor_I9 InterPro annotation	Add BLAST		59
Domain	103 – 345	Peptidase S8 InterPro annotation	Add BLAST		243

Fig. 3. The image above shows the results obtained from (www.uniprot.org) that gives information about the domains present in keratinase enzyme.

comprise mainly of alpha helical domains. Overall, alpha keratins make up the structural class of proteins, as they reside in the fibre cortex of hair. Second ones are beta keratins (8 to 25 kDa in size), which are non-extractable, less studies class of keratins. These are usually present in the hair cuticle and perform protective functions. Third ones are gamma keratins, being very rich in sulfur content, these keratins are approximately 15 kDa in size [2,5]. Their size is comparatively smaller than other classes of keratin. These keratins help to maintain the cortical super structure by crosslinking the disulphide bonds in the hair [2]. All these types of keratins can be degraded by the enzyme keratinase which belongs to a class of protease enzymes.

Proteases, also called proteinases or peptidases are the ubiquitous and found in all form of life from prokaryotes to eukaryotes to viruses. They are essential enzymes for cell growth, and can hydrolysed peptide bonds (proteolysis). Proteases are the largest group of enzymes, which classified into seven broad groups (Serine proteases, Aspartic proteases, Threonine proteases, Metalloproteases, Cysteine proteases, Asparagine peptide lyases, and Glutamic proteases) that can accomplish the proteolysis by completely different catalytic mechanisms. Alternatively, proteases can be categorized into three groups due to their optimum pH in which enzymes are highly active such as alkaline (basic), acid and neutral proteases [6]. Proteases which account for 60% of the world's marketed enzymes, is responsible for many applications such as detergents, food and leather processing [3].

Keratinases (serine proteases) are one of the most effective and striking component of proteases as compared to others. Initially, they were classified as proteases of unknown mechanism but in 1990s due to their high sequence similarity with alkaline protease that have keratinolytic activity, and restraint by inhibitors of serine protease, therefore, they were defined as a serine proteases [7]. Keratinase is one of the venerable enzyme to hydrolyze the proteins rich in disulfide bond like hair and has caused no or little damage to leather. Therefore, keratinolytic protease is the most suitable tool for

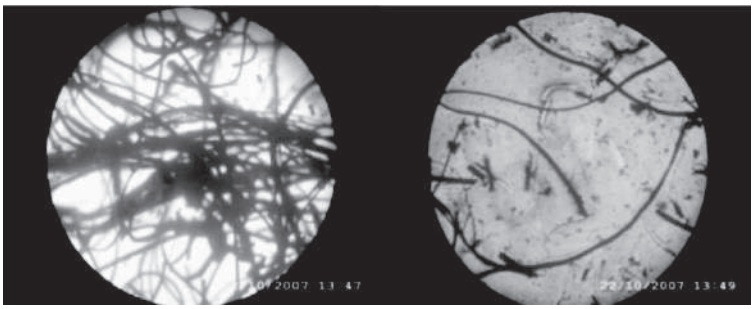

Fig. 4. This image shows how the structure of hair changes when it is treated with keratinase isolated from microbial sources [24]

various applications in industrial sectors [8].

1.1. Structure of keratinase enzyme

The enzyme keratinase (E.C. 3.4.99.11) is one of the serine hydrolases group that disrupt the disulphide hydrogen bonds in the keratin proteins [3,9]. According to uniport results, one of the protein keratinase produced by *Bacillus subtilis* contains two domains. First one is 59 amino acids long and encodes for inhibitor-I9; the other one is 243 amino acid long and encodes for peptidase S8. First domain occurs from 19 to 77 amino acid sequences and second domain occurs from 103 to 345 amino acid sequences (Fig. 3). The enzyme also has a metal ion binding site for calcium ion. This means that calcium ions act as the cofactors for keratinases; presence of calcium ions in the media can enhance the activity of keratinases. The structure of keratinase makes it very efficient in its function of degrading keratin proteins.

1.2. Why to employ keratinases?

Our daily green waste and animal waste includes plenty of keratins which remains undegraded due to their complexity. Such insoluble keratins may lead to environmental pollution if left untreated. So as a solution, such wastes are treated by keratinase enzymes which convert the waste into simpler as well as biodegradable substances [3]. One such example is shown in Fig. 4, that how the structure of hair changes when keratinase enzyme acts on it. The extracellular keratinases have been successfully isolated from several microbes by using several fermentation techniques and by optimising the conditions such as pH, temperature, and type of nitrogen and carbon source and the choice of microbe [9]. The keratinases from microbes are effective, biodegradable, economic, and provide much better results as compared to chemical treatments [10]. In this study, method of extraction of keratinase enzymes will be discussed and their industrial applications will also be considered.

2. DIFFERENT METHODS OF ISOLATION AND CULTIVATION OF KERATINASE ENZYME FROM NATURAL SOURCES

Work has been done since many years and keratinases have been isolated from different sources. Some of these methods for isolation and the sources from which keratinase have been isolated are discussed below.

2.1. Isolation from Waste Water Plant

A sample was collected from an activated sludge plant situated in a tannery and was taken to the laboratory for screening. For screening purpose, 2 g of agar was first dissolved in a solution of 10 mL fresh milk and 90 mL distilled water. After autoclaving, milk agar medium was poured into petri plates and inoculated with the sample taken from the activated sludge plant of the tannery. An incubation of 2 days at 30°C had shown the clear zone formation which indicated the presence of keratinolytic bacterium in the sample [3].

For growth and cultivation purpose, the medium of choice was feather meal medium whose contents were as follows in g/L: 0.5 NaCl, 0.5 NH_4Cl, 0.3 KH_2PO_4, 0.3 K_2HPO_4, 0.1 $MgCl_2.6H_2O$, 0.1 feather meal and 0.1 yeast extract with final pH of 7.0 to 7.5. The culture (5 mL) from overnight grown

pre-inoculum was added to 95 mL of this feather meal media and was incubated at 30°C at 150 rpm. Feather degradation was monitored by taking the OD of the media at 600 nm λ against distilled water as blank. Change in OD_{600nm} indicates that feathers being digested are a result of presence of extracellular keratinases acting on them [3].

2.2. Isolation from Bacteria

Keratinolytic bacteria were isolated using Nutrient agar medium having 1% (w/v) feather meal or skim milk. To check the presence of keratinophillic bacteria, soil (5g) diluted in distilled water (15 mL) by serial dilution followed by spread on agar medium and kept for 6 days incubation. Plates with clear zone around colonies will specify the presence of keratinolytic bacterial. After careful isolation, they were stored as glycerol stocks at -20°C [11,12].

A pre-isolated bacterial strains especially *Bacillus* spp. were used to cultivate the bacteria in fermentation media in order to produce extracellular keratinases. Basal salt media was used for the growth of this organism. The composition of this media in g/L was: 0.2 $CaCl_2$.H2O, 0.4 $MgSO_4.7H_2O$, 0.5 K_2HPO_4, 10 $NaNO_3$, 10 NaCl, 10 $NaCO_3$, 10 feather meal and 5 yeast extract. Pre-inoculum (3 mL) was taken and added to 97 mL of this media as 3% v/v inoculum. An incubation temperature of 45°C was given along with rotation of 180 rpm and the culture was incubated for two days following which the crude enzyme extract was obtained after centrifugation of the culture [13].

2.3. Isolation from Waste Dumps

A small soil sample was taken from waste dumps to the laboratory for scanning where it was diluted in distilled water and spread on to nutrient agar plates which were incubated at 37°C for 1 day. Once the colonies were observed, these colonies were taken and streaked on other agar plates which contained milk dissolved in it. Another incubation of one day was given at 37°C. Formation of colonies after incubation period indicates the caseinolytic activity of grown bacteria. For cultivation, 1% preinoculum was transferred to 100 mL nutrient broth having chicken feather meal as keratin source. The inoculated broth was incubated at 30°C in a shaking incubator at 150 rpm for 14 h. The resulting broth after incubation contained crude extracellular keratinase [14].

2.4. Isolation from Limestone Quarry

A limestone quarry was visited in India and some soil samples were taken from there. In the laboratory, these samples were dried and afterwards

Table 1. The table gives a brief account of species that have been reported to produce keratinase enzyme and the number of amino acids in each keratinase enzyme (source:https://www.ncbi.nlm.nih.gov/protein).

Sr. no.	Reported species	No. of amino acids	Molecular weight (kDa)	Accession number
1	*Bacillus subtilis*	362	37.22	AIY62812.1
2	*Bacillus sp. MKR1*	379	38.90	AEI59720.1
3	*Bacillus pumilus*	383	39.48	ACM47735.1
4	*Bacillus amyloliquefaciens*	382	39.15	AKR05134.1
5	*Stenotrophomonas maltophilia*	589	61.78	BAQ36632.1
6	*Geobacillus stearothermophilus*	546	59.68	AJD77429.1
7	*Bacillus velezensis*	382	39.15	AGC81872.1
8	*Bacillus thuringiensis*	347	36.94	APS24128.1
9	*Bacillus licheniformis*	379	38.89	AAY82467.1
10	*Pseudomonas aeruginosa*	301	33	6FZX_A
11	*Streptomyces albidoflavus*	360	36.4	AQX39246.1
12	*Actinomadura keratinilytica*	384	39.11	ASU91959.1
13	*Streptomyces sp. OWU 1633*	268	27.9	AAU94349.1
14	*Bacillus circulans*	383	39.48	AGN91700.1

crushed finely using a pestle and mortar to make a fine powdered sample. The solution (1%) of this sample was prepared by dissolving 1 g of sample in 99 mL distilled water and mixed properly. After giving incubation of 30 min at 25°C and 150 rpm, further serial dilutions were prepared from this sample. This solution was spread over the petri plates containing Horikoshi media. Successfully grown colonies were then selected for keratinolytic activity by streaking them on chicken feather media whose composition is as follows (w/v %): 0.5 KH_2PO_4, 1 feather, 0.5 Na_2CO_3 and 0.05 $MgSO_4$. Growth of colonies grown on this media had the keratinolytic activity [15].

For bacterial growth and cultivation of keratinase enzyme, chicken feather media was used in which feathers acted as keratin source and bacterial colonies grew producing extracellular keratinases. After inoculation of CFM with the sample, the broth was incubated at 30°C for 24 hours at 150 rpm. Feathers also acted as indicators as when they got degraded, production of keratinase was confirmed [15]. Work has been done on isolation of keratinase enzyme from several bacterial species. Characterisation of the isolated enzyme has been done and the number of amino acid per keratinase molecule has also been found out. Various bacterial species especially *Bacillus* spp. have the ability to produce keratinolytic enzymes (**Table 1**).

2.5. Isolation from Actinomycetes

Actinomycetes producing keratinases isolated from soil of poultry samples using serial dilution method. Soil sample (1 g) was suspended in distilled water (9 mL) and diluted up to 10^{-8}, prepared suspension was plated over AIA (with 5 mL L^{-1}glycerol) [16] and SCA of pH-7.3 [17] containing cycloheximide (50 µg mL^{-1}) as antifungal. The plates were incubated for 7–10 days at 28°C. By re-streaking, isolates were purified and screened. Pure culture of isolates were maintained ISP1 and stored in 20% glycerol stock at -20˚C for long term storage [1].

2.6. Isolation from Halophilic Microorganism

Keratinolytic halophilic microorganisms were isolated using serial dilution method. Halophilic medium for the isolation of bacteria containing peptone 0.2% (w/v), tri-sodium citrate 0.1% (w/v), yeast extract 1% (w/v), NaCl 10% (w/v), $MgSO_4$ 0.2% (w/v), agar 2% (w/v) with pH 7.5 was used. The medium with peptone 2% (w/v), glucose 6% (w/v), $MgSO_4$ 0.5%(w/v), yeast extract 1% (w/v), malt extract 1% (w/v), $FeSO_4.7H_2O$ 1% (w/v), NaCl 10% (w/v), K_2HPO_4 0.5% (w/v), and Agar 2% (w/v) was used for the isolation of fungi [18]. Bacterial plates were incubated for 24 h at 37°C and fungus plates were kept at 27°C for 6 days.

2.7. Isolation from Fungi

Mostly, research work has been done on several fungi which had been used as source to isolate keratinase enzyme. A brief account of ability of several fungi to produce keratinase enzyme has also been briefly mentioned in Table 2. The methods and sources of isolation discussed above are only few of all those studied yet. However, the prime focus of this study is enlightenment of significance of keratinase so we will discuss that how keratinases are used in several sectors.

Table 2. Different fungal species reported to produce keratinase enzyme (modified from Lange et al [46])

Sr. #	Genus	*No. of amino acids*	Substrate affinity	References
1	*Chrysosporium*	*C. keratinophilum, C. indicum*	Sewage sludge, hair	[47,48,49,50]
2	*Onygena*	*O. corvina, O. piligena, O. equina*	Feather, hair, bristle,hoof, horn	[47,51,52]
3	*Microsporum*	*M. canis, M. gypseum Microsporum*	Pig, sewage sludge, stratum corneum, nail, hair	[47,50,53,54,55]
4	*Arthroderma*	*A. gypseum, A. otae, A. benhamiae Arthroderma*	Sewage sludge, hair, horn, hoof	[47,50,56,57,58]
5	*Gymnoascoideus*	*G. petalosporus*	Hair	[48]
6	*Coccidiodides*	*C. immitis, C. posadasii*	-	[59]

7	*Aspergillus*	*A. fumigatus, A. oryzae, A. parasiticus, A. niger, A. flavus, A. terrus, A. sulphureus*	Poultry soil, feather, nail	[60,61,62,63,64,65,66]
8	*Trichophyton*	*T. rubrum, T. tonsurans, T. verrucosum, T. mentagrophytes, T. schoenleinii, T. vanbreuseghemii, T. terrestre, T. ajelloi*	Nail, skin, stratum corneum, sewage sludge, hair	[47,50,54,56,57,67-71]
9	*Paecilomyces*	*P. marquandii*	Nail and stratum corneum	[72]
10	*Talaromyces*	*T. trachyspermus*	Hair	[39]
11	*Scopulariopsis*	*S. brevicaulis*	Poultry farm and hair	[39,73]
12	*Doratomyces*	*D. microsporus*	Nail and stratum corneum	[72,74]
13	*Tritirachium*	*T. album*	Horn chips	[75]
14	*Myrothecium*	*M. verrucaria*	Feather	[76]
15	*Candida*	*C. albicans, C. tropicalis*	Feather	[57]
16	*Trichoderma*	*T. atrvoviride*	-	[77]
17	*Geotrichum*	*G. candidum*	Hair	[48]

3. STRIKING INDUSTRIAL APPLICATIONS OF KERATINASES

Keratinases can be solely utilized in the areas demanding harsh proteins degradation like nails, hair, feather, and also prions which can't be besieged by the conservative proteases [19]. Some of well-explored applications containing a theoretically huge market size have been particularized below.

3.1. Keratinous Wastes Recycling

The Keratinous wastes are chief by-product of the slaughterhouse, poultry, fur - and - leather processing industries, and are profusely spawned in numerous forms like hair, feather, hoof, horn, claws, nails, bristles, and wool [20]. The feather creates chief portion like poultry wastes along with 8.5 million tonnes round world and also the India subsidises 350 million tonnes over the year. Keratinous waste on the hydrolysis is transformed to the keratin hydrolysate that contains huge content of nitrogen, and is opulent in the hydrophobic amino acids. The characteristics of the keratin hydrolysate create it an extremely productive product along with the applications in various fields [21].

Numerous economically and effective methods are being developed merely for the keratin hydrolysates production. Unadventurously, keratin hydrolysates, particularly the feather meal have prepared only by the treatment along with an alkali, like KOH, NaOH, and $Ca(OH)_2$ at the great temperatures or the reduction along with the 2-mercaptoethanol in occurrence of the urea. The processing of the chemical may move to the degradation of heat-susceptible amino acids comprising methionine, tryptophan and lysine that may in produce non-nutritive amino acids, like lanthionine and lysinoalanine [22]. Because of the issues of the environment contiguous conservative chemical processes, the biotechnology through usage of the keratinolytic microbes as well as keratinases is promptly attaining ground. A miscellaneous group of the keratinolytic microbes have recognized that cultivate on or vitiate the keratinous wastes. It not only transforms waste to the productive hydrolysate, but also moves to concurrent manufacture of the keratinases.

Though, the processes have numerous restrictions like blockage because of unsolvable feather as well as the hair waste, unnecessary usage of the energy as well as lengthy incubation time merely for the degradation [10]. To compensate the restrictions, microbes-based process is being substituted merely by the enzymatic processes utilizing keratinases. Enzyme-based process is effective, reasonable, as well as time-saving.

The main limitation of utilizing the enzyme-

based process is manufacturing cost, though, which can be assuaged by the reusability of enzyme utilizing restrained measures. Nevertheless, widespread research requires to be completed on the reusability of the keratinases. Certain researchers have recognized the manufacture of feather meal utilizing keratinase restrained on the nanoparticles and by means of bio-restrained keratinases [15].

3.2. Medical Applications

The Keratinases are capable to invade skin as well as nail keratin, and therefore, discover application as preservative to enhance the effectiveness of the up-to-date drugs. They are being employed for numerous conditions of the skin for instance corn, callus and acne [19].

3.2.1. Keratinases used in trans-ungual delivery of drugs

Nail syndromes range from the comparatively inoffensive conditions such as pigmentation, to the debilitating as well as painful states at which the nail unit can be, hypertrophied, dystrophied, infected and inflamed. The most communal infection of fungi of nail, is onychomycosis, has a great pervasiveness rate along with roughly 700 million people anguishing from this situation worldwide. Maximum diseases of the nail are problematic to treat specifically ones having contaminations happen beneath nail plate. They necessitate a prolonged treatment period as well as face foremost issue of non-invasiveness, reoccurrence, and drug specificity, removal of the side impacts as well as enhanced compliance of patient [23].

Inopportunely, many of up-to-date medicines of the nail have restricted effectiveness because of pitiable permeability of drug through nail plate. Presently, numerous physical, chemical as well as mechanical methods are utilized for the trans-ungual distribution of current medications [24]. The mechanical methods, like nail avulsion as well as nail abrasion, are painful as well as invasive.

The physical methods involve hydration, carbon dioxide laser, occlusion as well as etching. The chemical methods comprise the usage of the keratolytic agents like thioglycolic acid, urea, salicylic acid and papain in amalgamation with the oxidizing agent like hydrogen peroxide. Chemicals like thioglycolic acid, N-2 mercaptoethanol, N-acetyl cysteine, mercaptoethanol, as well as N-acetyl cysteine are utilized at most of the times for degrading the surface of the nail as well as enhancing the permeation of nail plate. The compounds contain a powerful odour, which are acidic in nature, and comprise a prospective to respond with particular drug combinations [7].

The disadvantages of prevailing methods can be efficiently concerned by the usage of keratinases. Keratinases as the molecular scissors are splitting the hard keratin protein that establishes chief portion of the nail plate, thus slackening plate as well as improving trans-ungual permeability of the drug [25]. Keratinases occur on mutually intercellular matrix which connects cells of nail plate together and also dorsal nail corneocytes merely by disintegrating surface. Keratinase merely from the *Paecilomyces marquandii* was exposed to incompletely disturb nail plates as well as enhance the permeability of the drug. A multifaceted subtilisin-γ-glutamyl transpeptidase, for instance, keratinase KerN has been explained to increase delivery of the drug via nails. Moreover, an insufficient keratinase-based marketable preparation involving FixaFungusTM, Pure100 Keratinase as well as Kernail-Soft PB, are obtainable in market only for considering nail disorders [26].

Besides infections of nail, the keratinases can be used merely for the permeabilization of the skin tissue to improve the delivery of the drug over surfaces of the skin. The keratinolytic agents eliminate the hyperkeratotic scratches, refining the introduction of inflamed surface of the skin to the current drugs [27], established skin agent merely by restraining keratinase to the porous sheet that releases skin as well as recovers the permeability of the drug. To feat the probable of the keratinases like well-organized ungual enhancers as well as the permeabilizers of the skin tissue, widespread research wants to be assumed. Keratinases can develop an important preservative of prevailing nail lacquers. Absence of appropriate in vitro approaches to measure the degree of permeation of the drug is primary exertion. More premeditated approaches accompanied by human as well as animal trials want to be supported out for the commercialization of the keratinase-created drug delivery preparations [22].

3.2.2. Calluses and Corns Removal

Calluses as well as Corn, also known as hyperkeratosis, are excruciating thickenings of the weakened skin which frequently form the dorsal surface of fingers as well as toes. During treatment, podiatrists recommend the usage of keratinolytic agent like salicylic acid. It softens keratin which creates the chief portion of the corn as well as dense layer of the weakened and dead skin [28]. The usage of the keratinases is natural as well as greener substitute to usage of the salicylic acid. Numerous groups related to research have discovered usefulness of the keratinases merely for eradicating horny skin layer.

3.2.3. Treatment of acne

Acne is a mutual skin problem which happens because of hindering of sebaceous gland by occurrence of unwarranted keratin. Like keratinases can liquefy departed cells as well as the keratin which blocks sebaceous glands, this can be functional to the acne treatment. A keratinase-related product which can be effective adjuvant in the acne therapy has been untested since 2001 [10].

3.2.4. Bio-safety to prions that are infectious

One of the greatest authoritative applications which have revolutionized the keratinase research is in field of the decontamination of the prion. Prions are the transferable agents which cause lethal as well as communicable brain diseases [5]. These transferable biomaterials are evolving contaminants related to the environment that are emitted into environment over many routes for instance disposal of the mortalities, body fluids, PrPSc polluted effluents from the hospital, slaughterhouse as well as research facilities. It amasses via ingesting of the meat as well as reprocessed the waste products for instance bone meal of infested animals. Straight passageway of PrPSc from environment towards host happens by inhalation or ingestion while secondary passage happens via medical devices specifically the stereotactic electrodes. Disposal as well as storage of the biological and clinical wastes is the stuff of worldwide concern which must be distributed with immediately [26].

Numerous physical and chemical approaches of sterilization and disinfection safeguarding the decontamination of prion have been deliberate widely by Weber et al [29]. Though, the prion disinfecting methods are not only eco-unfriendly, punitive, and also the energy intensive, but also do not guarantee to comprehensive damage of contamination. Moreover, their constant application can harm the medical related devices, thus, warning their usage in the hospitals. To cope with degradation of infectious the domain of the prion proteins, enzyme-related practices could be an innocuous resolution [30].

Keratinases are talented candidates for the decontamination of the prion like they break β-keratin with improved rate than the conservative proteases, on account of structural similarity of extremely accumulated β-pleated structure of the prion protein (PrPSc) of feather, abundant in the β-keratin. Cheng and co-workers [31] were the first one to determine the dilapidation of the PrPSc infested brain tissues by the KerA of *B. licheniformis* PWD-1. Numerous keratinases from assorted group of actinomycetes as well as the bacteria have been exposed to damage prion proteins in the lab experiments. Keratinases from the *Nocardiopsis* sp. and *Streptomyces* sp. TOA-1 have been recognised to destroy the amyloid prion proteins nevertheless under dangerous situations for instance alkaline pH, as well as high temperatures. Additionally, limited keratinases from the thermophilic organisms for instance genus *Thermosipho*, *Thermococcus* and *Thermoanaerobacter* have been damaged PrPSc deprived of pre-treatments, and also have been working for emerging prion free as well as non-pathogenic animal meal. Moreover, a collection of serine proteases generate from the lichens *Parmelia sulcata*, *Lobaria pulmonaria,* as well as *Cladonia rangiferina*, and also keratinase from *B. licheniformis* have exposed the prospective to damage transferable prion proteins underneath slight conditions. Recently, a profitable product specifically Pure100 Keratinase propelled by the Proteos Biotech has been engaged to disinfect surgical instruments which are vulnerable to contamination of the prion [32].

Nevertheless, many enzymatic approaches want to be functional in amalgamation with the other treatment regimens such as alkali, detergents, or high temperatures. The regimes are normally dangerous and environmentally unfavourable. Therefore, search endures for recognizing catalytically additional detailed enzymes which can

abolish infectivity of the prions during reasonable conditions deprived of denaturing pre-treatment stages [5].

3.3. Keratinases as Efficient Substitutes Compared to Commercial Proteases

Keratinases contain conservative proteases along with their probable to damage both insoluble as well as soluble proteinaceous substrates, and also may demonstrate to be improved than predictable proteases in the protease-prevailing sectors.

3.3.1. Keratinases as feed additives

The animal feed, frequently made of vegetable as well as cereals proteins together with the products of meat are problematic to assimilate as well as digest by the animals. The utilization of feed can frequently be enhanced by accumulation of the enzymes to feed. A great array of the feed enzymes for instance lipase, xylanase, cellulase, α- amylase, β-glucanase, pectinase, protease, and phytase are in market like additives of the feed in the diets for poultry, fishes, cattle, sheep and pigs. The usage of the proteases is recognized for the quicker development as well as performance of the young ones by enhancing nutritional value as well as digestibility of prevailing dietary proteins.

Keratinases contain originate their location in the feed enzymes that keratinases can cleave the PrpSc proteins, and also create the products of the meat innocuous for the ingestion by the animals. The Keratinolytic proteases have been fine recognized to provide improved consequences by accompanying meat as well as cereal-related diets. They had affirmative impacts on performance of the growth to the young ones, enriched the utilization of the amino acid as well as the structure of the gut villus. Moreover, enhanced the consumption of the keratinase accompanied diets has moved to lessening in the requirement of the feed [33].

Keratinase derived from the *B. licheniformis* PWD-1 advanced in the trade name Versazyme is utilized like the nutraceutical product and also has move to momentous enhancements in the performance of the broiler. The accumulation of the Versazyme in pelleted as well as mashed diets exposed valuable impact on initial growth and feed utilization of broilers. Additional keratinase of the *B. licheniformis* PWD-1 promoted under the product name Cibenza DP100TM is utilized for maintainable as well as fruitful growth of the piglets [31, 34].

3.3.2. Detergents

The proteases have been extended to industry of the detergent merely for supporting in the proteinaceous stains removal. Nevertheless, keratinases are supposed to require improved detergency like they are wide-range proteases along with restored substrate specificity merely for both the insoluble as well as soluble substrates of the protein. They can simply hydrolyze immobile proteins upon surface, and it can also eradicate stains involving keratinous soiled blood stains, cuffs as well as collar. Additional forthcoming application of the keratinases is in field of the wiping up the drain pipes as well as outlets stopped with the hair as well as additional keratinous matters. Itsune et al [35] have established scrubbing agent conformation organized by the compounding keratinase as well as the non-sulfur reducing agent which is able to wiping smarmy matter committed on drain outlet of bathroom and the dirt triggered by the pollutants for instance hair as well as scales, along with huge effectiveness in innocuous manner. The profitable product such as BioGuard Plus is accessible which integrates a mixture of diverse enzymes involving keratinases merely for wiping out drain pipes as well as tanks [2].

3.3.3. Leather dehairing

The processing of the Leather comprises four chief stages viz. dehairing, soaking, tanning as well as bating. Conservatively, the soaking is achieved utilizing the alkali, the dehairing is the sulfide-related, although tanning as well as bating includes the usage of solvents, lime and salt. The usage of the punitive chemicals creates the industry of the leather exceedingly polluting which subsidises to foremost disposal problems of the effluents [14].

The usage of the enzymes is greener substitute moving to lessening in the pollution in the environment and also enhanced quality of the leather. Many profitable proteases for instance Pyrase, NUE, Clarizyme, as well as Aquaderm are accessible that are utilized in dehairing, bating as well as soaking. Nevertheless, the comprehensive replacement of the chemical processes merely by enzymes is not informal because of economics

of enzyme-related processes. This matter can be concerned by usage of the proteases which have improved speciality to the hair, and also are more effective catalytically and will be obligatory in slighter amount [36].

Moreover, the keratinases wanting the collagenolytic, and containing slight elastolytic happenings are progressively being discovered due to the dehairing process. The enzymes would assist in discerning interruption of the keratin tissue in follicle, thus, dragging out complete hair deprived of distressing tensile strength of the leather. Like for industry of the leather, the keratinases derived from the *Aspergillus nidulans*, *Bacillus subtilis* S14, *B. subtilis* KD-N2, *Bacillus sp*. PPKS-2, *Paenibacillus woosongensis* TKB2, *Trichoderma harzianum* MH-20 displays extraordinary dehairing competences deprived of the collagen deprivation. An enzyme-related dehairing entirely eradicates the want merely for the toxic sodium sulfide in the leather processing. This environment-friendly dehairing method contains most significant compensations, primarily on the ultimate quality of the leather product, and overwhelming pollution problems in environment produced by conservational chemical processes, and also products of the waste [21].

3.3.4. Textiles

The wool is mechanical protein fibre considered by great degree of the cross-related disulfide bridges (S–S) which discuss the mechanical resistance as well as strength to the deprivation by the proteases. It is accredited to overlying layers of the cuticle categorized by exo-, epi-, and also endocuticle wrapping the external surface of fibers. Epicuticle is abundant in the lipids although endocuticle as well as exo- are encompassed of the keratin, abundant in the disulfide bonds [23].

The structure creating cuticle plays significant part in felting contraction of fibers in washing and disturbs the dyeing. Another the Chlorine-Hercosett process, including the usage of absorbable organic chlorides (AOX), has been engaged in processing of the wool over the last 30 years to switch the felting contraction of the wool fibers. Nevertheless, this treatment moves to weight damage of fibers with the clearance of dangerous chemicals in environment. The other chemical related important concluding progressions, comprising scrubbing (cleaning), dyeing or bleaching, cause expulsion of a dissimilarity of poisonous chemicals. Moreover, the procedures are not only time-consuming as well as energy intensive, but also have a propensity to damage the simple material. The usage of the enzymes is pragmatic as an eco-friendly safe substitute as well as numerous protease-related commercial products for instance Savinase, Esperase, Alcalase, as well as PeriZyme Tuggumm Type EX have been discovered in diverse textile concluding progressions over the last decade. Nevertheless, in accumulation to eliminating cuticle, proteases infiltrate deep in wool fiber thus destructing it and moving to damage of tensile strength as well as the weight of the fibre [7]. This issue has been assuaged by enhancing molecular weight of the proteases via alterations of chemicals using polymers for instance glutaraldehyde, Eudragit S100 or PEG. Utilizing keratinases that would selectively mark scaly keratinous layer of wool deprived of harmful other portions of fiber may be anticipated substitute. Numerous keratinases derived from the *B. licheniformis* L11, *B. thuringiensis*, *Pseudomonas sp*., *Bacillus cereus*, *Fusarium* sp., and *Stenotrophomonas maltophilia* DHHJ have been familiar to increase stroked-shrink hostility dyeing deprived of harm of fiber weight. The action of the keratinases has been enhanced additional by coalescing them along with lipase or cutinase. The strength fibre has been enhanced by employing transglutaminase [37].

Therefore, keratinases unaided or in amalgamation with the other enzymes can help in emerging necessary formulations for better-quality wool processing. The raw silk wants degumming to eliminate fibrous protein, sericin which strengthens the fibroin fibers self-possessed, thus as to afford the soft feel as well as fibers luster. This method is significant for following dyeing. Conservative degumming methods are treated along with the soap, oxidizing agents and alkali at greater temperature in agitation. These situations change the chemical as well as physical properties of the fibers moving to dilapidation of main material of the silk. Enzymatic behaviours of proteases for instances Papain Degummase, Pepsin Trysin, Savinase Alcalase, Protease N Amano, Protease A Amano, Palkobate and Protease M Amano are in emphasis over the conservative method. Though, many proteases are considered by less degree of

specificity to the sericin. Therefore, enzymes along with improved specificity are necessity of hour as well as keratinases along with their extensive substrate range may verify to be practicable substitutes [38].

3.4. Prospective applications of keratinases

Along with the well-recognized application fields, keratinases comprise impending to be utilized in most of the areas providing selective deprivation of rough proteins such as hair as well as skin. Researchers have begun discovering some innovative applications like hair as well as cosmetic preparations, the elimination of the earwax, and also pearl bleaching.

3.4.1. Earwax removal

Earwax that is also referred to the therapeutic term cerumen, is hydrophobic defensive covering in ear canal of the humans and also other mammals. Earwax principally comprises of the shanty layers of the skin, along with 60 % of the earwax comprising of keratin. Cerumen accumulation is reacted by the softeners comprising cerumenolytic agents involving sodium bicarbonate, glycerine, arachis oil, carbamide peroxide, dichlorobenzene, triethanolamine, turpentine, hydrogen peroxide, and urea. Nigam [39] has revealed configuration for eliminating the human cerumen which comprises bicarbonate in aggregation with amalgamation of the enzymes involving protease/ keratinase, amylase and also lipase. They have discovered the opportunity of utilizing enzymes like Trypsin, Pancreatin, Collagenase, Subtilisin, Carboxypeptidase, Keratinase, Papain, Bromelain, Elastase and Aminopeptidase. The enzyme-related cerumenolytic configurations are commercially safe feasible and efficient in removal of the cerumen from external ear canal. The huge conc. of the keratin in the cerumen creates keratinase/ keratinolytic protease a very possible candidate to be discovered additional for claim [19].

3.4.2. Pearl bleaching

Pearls are designed like a defence appliance in contradiction of possibly intimidating irritants in the living shelled molluscs like an oyster. The organic constituent known as “Mother of pearl” or “Nacre” is concealed over interfering irritant. Nacre is made chiefly of the crystallized calcium carbonate and also conchiolin, black coloured organic protein. In pearl establishment, the organic impurities like able cells of the mother of the pearl oysters, mucilage as well as necrotic portion of the mantle tissue pieces are surrounded in pearls and they want to be handled to improve their quality of the gem.

The Pearls are exposed to bleaching behaviour which assists in blanching them, evening out the irregularities of the color and overwhelming brown color of the conchiolin. The methods of bleach utilized are very slight like hydrogen peroxide; permitting mild lightening of pearl nacre deprived of detrimental the quality since surface of the pearl is polluted with organic impurities such as mucilage, tissues, as well as cells. Zhang et al [40] have discovered the usage of keratinases in bleaching of the pearl. Keratinases can be utilized in preliminary treatment to eliminate keratin impurities on pearl surface monitored by conservative bleaching or processing methods [28].

3.4.3. Cosmetics/personal care products

Keratinases have been engaged in formulation of the cosmetics for hair as well as skin. For the skin, keratinases have been further in configurations for whitening of the skin, dispelling of the freckle, and also bleaching. Keratinases can be utilized for the removal as well as exfoliation of the stratum corneum. Keratinases have been further to compositions hair for instance conditioner, hair gel, and shampoo where they perform a double role in refining color of the hair, luster and quality with concurrent cleaning as well as elimination of layers followed on hair [13].

3.4.4. Processing of edible bird’s nest

Nests constructed by an insufficient species of the swiftlets are spent by the humans internationally, like great-value fragility or like medicinal Applied Microbiology Biotechnology food. Nests are comprised of gelatinous constituents, and comprise intertwined feather and also fluff like impurities. They are cleaned by the processes for instance hand picking, sieving as well as hot water treatment which are time-consuming and also unproductive. The huge cost or demand for the nests has moved their constructers to approve some dangerous practices like reacts with the silicates as well as peroxides. Plumages as well as feather are chief impurities, formulations related to specialty

enzymes like keratinases, which can selectively spasm insoluble proteins may demonstrate to be an efficient method of the processing the nests. Nevertheless, widespread research wants to be lead to authenticate this, and also an enzyme has to obtain GRAS status to be oppressed for the application of the food [4].

3.5. Other applications

The application areas emphasized above practice the chief contribution of protease market. Moreover, of these, proteases are utilized to smaller extent in fields like the synthesis of peptide, elimination of the silver from the photographic films and also contact lens cleaning. More intensive research wants to be completed to damage the intrinsic advantages of the keratinases over the proteases in sectors.

4. DISCUSSION AND LATEST TRENDS

In recent time, keratinase is the most noteworthy member of proteases group of enzymes that can effectively hydrolyse the tough insoluble protein and polypeptide molecules into amino acids [11,41]. Keratinase are ubiquitous in nature but as compared to fungal, animals and plants, bacteria are the attractive source of keratinolytic enzymes as they can be cultivated easily in lab and produced industrially important enzyme in large amount in a short time by optimized fermentation methods. Keratinolytic enzymes are produced by organisms only in the presence of keratin substrate. Bacterial protein has long shelf life without reduction in activity and can be stored easily [42]. Naturally, bacterial keratinases are extracellular and soluble protein. They are secreted directly into the fermentation culture broth, so the downstream processing of these extracellular keratinolytic enzymes are easy as compared to the enzyme obtained from other sources [41].

Keratinase have great sequence homology with alkaline proteases and these are catalytically active in neutral to alkaline pH range and nearly thermophilic temperature, which is the most prominent feature of alkaline proteases. Therefore, keratinolytic enzymes are define as serine proteases which belong to alkaline proteases (EC.3.4.21-24, 99), which are either have a metallo-type or serine centre. They are widely used in food, detergent, leather, and pharmaceutical industries [42,43]. So far, the production of keratinase from poultry soil bacteria has been discussed and applications of the enzyme have also been illuminated.

Despite being successfully applicable in many emerging fields, scientists are still finding new ways to make keratinase more efficiently applicable. One such example is isolation of keratinase from *Meiothermus taiwanensis* WR-220 which is a thermophilic bacterium and shows maximum keratinase activity at temperature around 65°C [44] providing an eco-friendly way to convert keratin wastes to valuable amino acids. Since keratin is very difficult to degrade and using enzyme hydrolysis method assisted with high temperature can provide very efficient keratinolytic activity. Moreover, the gene for keratinase enzyme from *Meiothermus taiwanensis* WR-220 was ligated into an expression vector and further transformed to a bacterium to further enhance its expression [44].

In another research, a chicken feather-degrading bacterium *Fervidobacterium islandicum* AW-1 was isolated from a hot spring of Indonesia. The optimum temperature for the growth of this bacteria was 70°C and it could degrade chicken feathers. The crude sample of extracellular enzyme extract obtained by the fermentation of this bacteria hydrolysed keratin efficiently at 90°C [28] This reveals that using high temperatures for keratin degradation can provide best results when compared with the results of degradation at lower temperatures i.e. 35 to 40°C by mesophilic bacterial keratinase. Such thermostable keratinases can provide quick and efficient dehairing in leather industry.

Experiments are also done using keratinolytic fungi including *Fusarium* sp. strain 1A, *Trichophyton* sp., *Cladosporium* sp., *Chrysosporium* sp, *Microsporum* sp., *Trichoderma* sp., and *Phytophthora* sp. isolated from the soils where there were keratin deposits [32]. Horse hair were provided as source of keratin which is a substrate for the keratinase enzyme. The structure of hair was observed over a period of several days under SEM to check the effect of keratinase and later it was revealed that *Fusarium* sp. strain 1A, *Microsporum sp. and Chrysosporium* sp. have the greatest ability for degradation of keratin [32].

The growing interest in thermophilic microorganisms and their potential biotechnological applications explains the increasing number of studies in extremophilic microorganisms around the world [45]. Thermophilic keratinases are being isolated from different regions of the world and are being employed for several purposes. The only reason is that when we need to degrade keratin, a higher temperature helps to weaken the bonds within a keratin molecule and afterwards enzymatic hydrolysis becomes much more efficient and quick. So, the use of thermophilic bacteria for isolation of keratinase rather than mesophilic bacteria have become the choice of the scientists as per the latest researches [45].

5. CONCLUSION

With growing emphasis on the eco-friendly atmosphere, and use of biocatalysts in industrial processes gained significant attention in this era. There is a great need to search new industrially relevant enzymes from microorganisms that have abilities to accomplish demand of industries. Keratinases are the useful industrial enzymes, we have so far discussed most of the applications of keratinases and their major roles in upcoming biotechnological era. The use of keratinases in previously mentioned fields can solve many problems such as reuse wool waste, poultry feather waste degradation, used as bio-control (keratinases use against plant pathogens because of having antagonistic activities), in detergents, has bio-fertilizing potential and have various agricultural applications. The usage of bacterial keratinolytic enzymes to improve the production of agricultural crop has developed as an alternative and sustainable tool to meet challenges. In these days, scientists have focused to use organic (composted) wastes as fertilizers that will decrease the prices of chemical fertilizers (commercial fertilizers) and it will be more favourable for our ecosystem. Hence, keratinase is a productive and economic choice for industrial and biotechnological applications.

6. CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests. We assure the quality and integrity of our work. This study is completely independent and impartial; all points taken from other authors are well cited in the text.

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Review Article

Methods to Analyze Proteins from Soils

Salma Mukhtar, Nayaab Laaldin, Samina Mehnaz, and Kauser Abdulla Malik*

School of Life Sciences, Forman Christian College (A Chartered University), Lahore

Abstract: Recent advances in molecular techniques, especially system approaches at DNA, RNA and protein levels have opened an emerging field in microbial ecology. These approaches can be used to identify the specific microbial genes and their functions directly from environmental samples. Among the different 'omics' approaches, metaproteomics is used to study microbial ecology and it plays an important role in the determination of microbial functionality. It provides detail about the structure and function of microbial populations from soil samples. However, proteins isolation and purification is very challenging due to complexity of soil samples. Only a few methods give high quality of extracting proteins and other methods can only be used to separate proteins using SDS electrophoresis but they are unable to characterize and identify specific proteins present in a soil sample. This review has mainly focused on recent advances in metaproteomic strategies to understand the structure and function of soil microbial communities. Three methods for protein extraction from soil samples were explained here, e.g., (1) using the phenol extraction method, (2) cell lysis method using different concentrations of SDS and alkaline lysis method using NaOH. For purification and identification of proteins, HPLC, FPLC, 2D-LC, LC-MS, MALDI-TOF and shotgun proteomics analyses were explained.

Keywords: Metaproteomics, Halophiles, 2-D Electrophoresis, High performance liquid chromatography (HPLC) Mass spectrometry (MS).

1. INTRODUCTION

Soil represents a naturally occurring complex system in which different biological, chemical and physical components interact with one another. A specific level of each component is maintained by formation, transformation and decomposition of complex organic materials in soil into simpler available nutrients by soil microbial populations [1-3]. Some DNA and RNA based methods have been regularly used during 1990s to characterize and identify the function of different microbial proteins and enzymes from soil samples. For example, micro-autoradiography and *in situ* hybridization had been used as powerful tools to study function-based microbial diversity and to identify the specific protein-protein and protein-substrate interactions in individual bacterial cells from soil microbial communities [4, 5]. By using stable isotope probing (SIP) DNA or RNA molecules, bacterial species involved in bioremediation of toxic compounds can be identified [6, 7].

Recently, various meta-omics approaches such as metagenomics, metatranscriptomics and metaproteomics have been used to study microbial ecology and functional make up of natural environments [8, 9]. 'Metaproteomics' is defined as the characterization of whole microbial protein complement from an environmental sample at a specific time. Microbial communities from different environments such as soil, marine and fresh water and activated sludge have been studied by using metaproteomic approaches [10-13]. A number of experiments have been performed for proteomic analysis of individual cells or microbe, protein-protein interactions and identification of disease biomarkers, but metaproteomics technique can be used for the entire environmental sample at a time to study functional microbial community [14-16]. Metaproteomic based techniques can be used for identification of different microbial proteins and enzymes with potential biotechnological applications such as biodegradation of complex organic pollutants, biological nitrogen fixation and

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*Corresponding Author: Kauser Abdulla Malik; kausermalik@fccollege.edu.pk

other environmental processes [17].

In proteome analysis from soil samples, to get high quality proteins is a very critical step because protein distribution varies with change in microbial populations, e.g. Gram-negative bacteria have variety of intracellular proteins while Gram-positive bacteria have a great variety of extracellular proteins [18, 19].Some other methodological challenges may be there to get high quality and maximum number of proteins from a complex soil samples. Physical characteristics of soil, such as salinity, pH, temperature and texture, microbial diversity and presence of high amount of extracellular enzymes may affect the protein extraction process [20] and ultimately hinder the expression of proteins profiles and characterization of microbial metabolic pathways in a specific soil sample [21].

A number of previous studies have discussed on importance of metaproteomics from different environments. Metaproteomics studies on the plant rhizosphere microbiome help to understand complex metabolic pathways, detection of multiple functions of microbial genes and proteins. This review has focused on the recent advances in soil metaproteomics analysis, such as identification of proteins through HPLC, 2D-LC, MS/MS and MALDI analysis.

2. SOIL SAMPLE PREPARATION

Soil is one of the most complex sample types regarding metaproteomics analysis. It contains organic matter, inorganic ions and complex microbial communities. Microbial diversity identified from the soil is more diverse and complicated as compared to diversity other samples such water, human, animal or plant tissue [9, 11]. It is very tricky to handle soil samples during metaproteomics analysis as they react very quickly to environmental changes. Proteins extraction from soils is especially difficult and critical because it is mostly clay soil with more salt concentrations and less permeable than the loam and sandy soils [22, 23].

3. PROTEIN EXTRACTION

Extraction of total proteins from an environmental sample especially from soil is very important for metaproteomics analysis [24, 25]. Lysis of Gram-positive bacteria such as *Bacilli* and *Cyanobacteria* is difficult as compared to Gram-negative bacteria. So, protein concentrations are low because of incomplete lysis of Gram-positive bacterial cells. Soil sample also contains humic substances which cause problems in the protein extraction process. These compounds mostly interfere with protein purification and estimation through colorimetric methods and SDS-PAGE analysis [26]. Some organic substances present in soil samples hindered the separation of individual peptides obtained by tryptic digestion [27]. Whiffen et al. [28] suggested that humic acid and polyphenolic compounds usually interfere with protein estimation from soil samples when the Bradford assay is used (Fig. 1). Three different methods have usually been used for protein extraction from soil samples: (1) by using ‘phenol method’ in which, lysis buffer with phenol used for cell lysis and extraction; (2) ‘cell lysis’ by using different concentrations of SDS; (3) protein extraction by cell lysis with alkaline solution e.g., NaOH. Recently, different kits have been used for extraction of total proteins from soil samples, e.g., Power Soil Protein kitand FastProtein™ with blue and red matrix. Soil protein extraction by using these kits is relatively easy and protein concentration is good as compared to previously described methods [29, 30].

4. PROTEIN PRECIPITATION

To concentrate and purify, protein samples are precipitated by using various inorganic or organic compounds. Salting out of some neutral salts such as ammonium acetate, and ammonium sulfate have been commonly used for protein extraction by protein-protein interactions [31, 32]. In this technique, to change the charge on the surface of proteins, salt concentration in solution is increased so that hydrophobic parts of proteins interacts and proteins can be precipitated from solutions easily.A number of previous studies reported that protein can be precipitated from soil samples by using ammonium acetate buffer[33]. Some organic solvents such as methanol, ethanol or acetone can be used for precipitation of proteins (Fig. 1). In this method, temperature is considered an important parameter to avoid denaturation of proteins. Some previous studies showed that protein precipitation

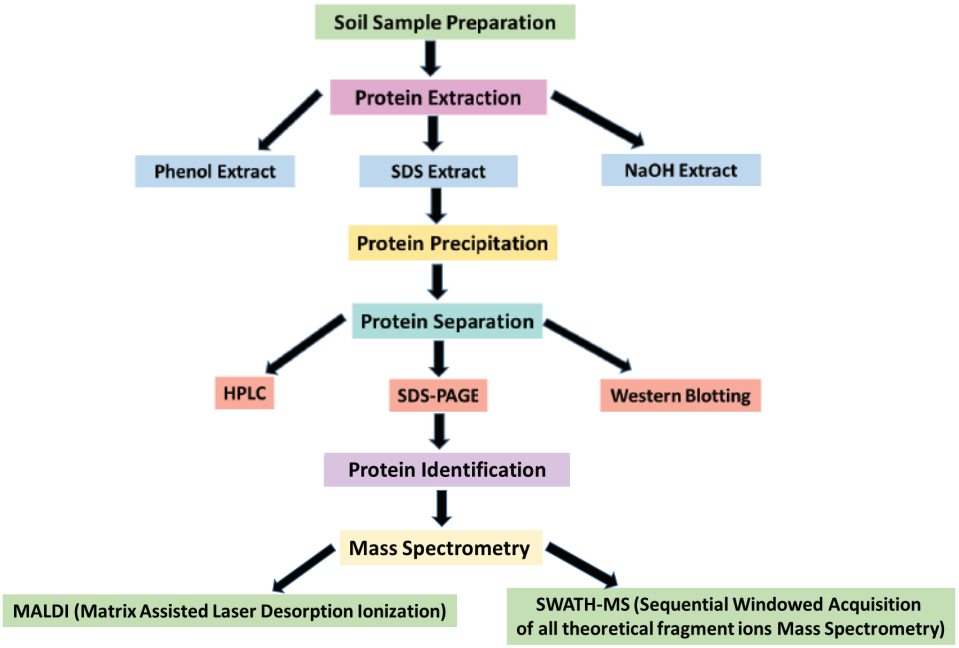

Fig. 1. Overview of soil metaproteomics strategies

has also been done by using polymers, such as polyethylene glycols and dextrans. This method has been used for proteins identification from biomaterials [34].

5. PROTEIN SEPARATION

Protein separation techniques play important roles in the development of metaproteomics of soil. For protein separation, a number of methods have been used, such as polyacrylamide gel electrophoresis (PAGE), western blotting and high performance liquid chromatography (HPLC).

5.1. SDS-PAGE

This technique is mostly used to separate and characterize different proteins from a mixture. Different proteins from a complex sample such as soil can be purified, analyzed and identified by using polyacrylamide gel electrophoresis (Fig. 1). Initially blue-native (BN-PAGE) has been used to separate a mixture of proteins [35, 36]. To address the resolution of complex mixtures of proteins, SDS-PAGE has been introduced. Various chemicals like detergents are used to denature the proteins which bind to individual proteins and help them to separate according to molecular mass [37, 38]. SDS-PAGE or one-dimensional electrophoresis has commonly been used to separate proteins on the basis of their molecular mass. Sodium dodecyl sulphate (SDS), a detergent is used in this method to denature the protein, so that they can be purified easily [28, 39]. It is a native technique which has been previously used to isolate and study enzymes and other proteins. In two-dimensional electrophoresis (2-D electrophoresis), proteins are separated in two directions: according to their isoelectric point in the 1stdimension and SDS-PAGE in the 2nd dimension to separate proteins on the basis of their molecular weights (Fig. 2). This technique has the main advantage of identification of proteins with some post-translation modifications [40, 41]. Now 2-D electrophoresis is widely used to study the expression profile of proteins both quantitatively and qualitatively (Fig. 1 and 2). The intensity of spots provides the information about the presence and absence of proteins expression. A number of softwares have been used to analyze complex images [42]. The main drawback of this technique is to study and characterize proteins with more hydrophobic parts, e.g., membrane proteins. Another problem associated with 2-D electrophoresis is the analysis of proteins with low abundance. This technique has been used to analyze and separate more complex and less purified proteins from a mixture. SDS-PAGE is used to measure the protein size and molecular weight of proteins, peptide mapping, estimate protein purity, comparison of the polypeptide composition of

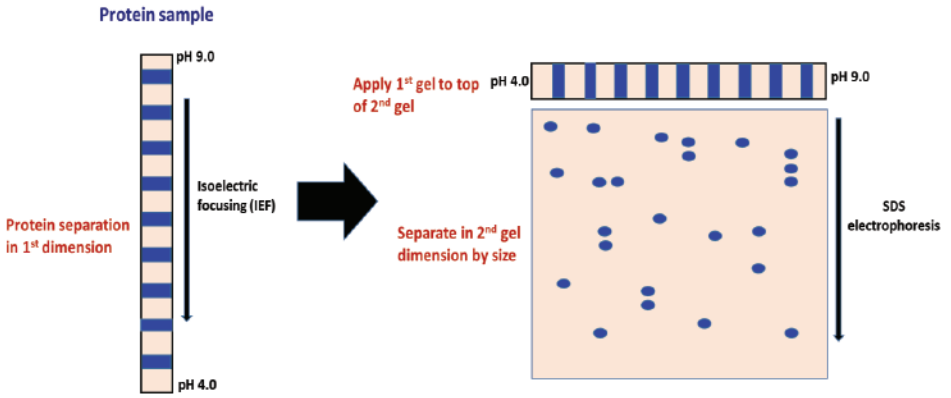

Fig. 2. Separation of different proteins from a sample by using 2-D SDS gel electrophoresis

different proteins and ubiquitination of proteins [43, 44].

5.2. High Performance Liquid Chromatography (HPLC)

It is highly dynamic technique in its nature especially depending on physiological conditions and abundance of proteins that are analyzed at the same time (Fig. 1). Same proteins show different expression in different cells and tissues [45, 46]. Reverse phase high performance liquid chromatography is the most commonly used technique which has been used for the separation, quantification and identification of peptides, proteins and other small organic molecules on the basis of their hydrophobicity. By using this approach, small molecules can also be detected with the application of high pressure on the rate of solvent flow in the separation process [47, 48]. In HPLC technique, different detector types are used to separate and identify all proteins but for identification of individual peptides, HPLC is not sufficient by only using UV spectrum [49]. Duration of separation of specific proteins is controlled by using a high-pressure pump and computerized system [50, 51]. HPLC is also used for identification of proteins with post-translational modifications. For this purpose, HPLC system has water as mobile phase for the accurate detection of peptides with post-translational modifications [52, 53].

5.3. Fast Protein Liquid Chromatography (FPLC)

It is a type of medium pressure liquid chromatography that can be used to purify proteins with high resolution and reproducibility. The distinctive feature of this technique is the stationary phase with small beads packed in plastic or glass columns which have high loading capacity [54]. The most common forms of FPLC are ion exchange, affinity and gel filtration chromatography. This technique can be used to purify different proteins and enzymes with applications in agriculture, industry, medicine and bioremediation of complex organic compounds [55].

5.4. Two Dimensional Liquid Chromatography (2D-LC)

The two dimensional liquid chromatography (2D-LC) is usually used to analyze two samples of separate liquid chromatographs for combined data analysis [56]. This chromatography technique can be used to analyze and separate complex mixtures with lots of proteins such as soils, liquid samples, e.g. blood, urine, waste and marine water [57]. The 2D-LC has important applications in proteomic and metabolomic studies of various environmental samples which are involved in the identification of targeted and non-targeted proteins [58].

5.5. Western Blotting

Western blotting is a technique used to study different proteins from a mixture or peptides from an individual protein. This technique can be applied for identification of proteins, protein-protein interactions, the kinase activity of proteins, cellular localization, monitoring of post-translational modifications, e.g., glycosylation, methylation and ubiquitinylation [59, 60]. Analysis of some proteins

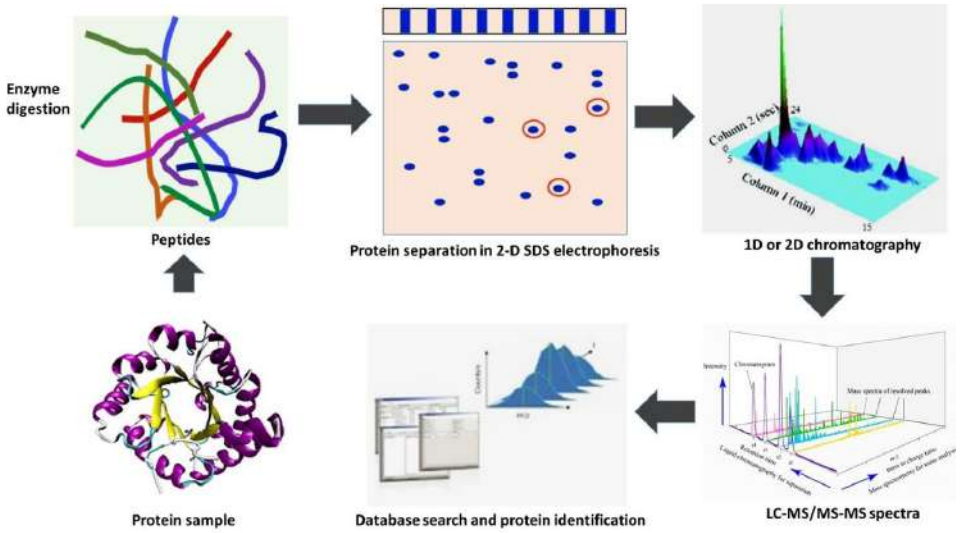

Fig. 3. Identification of proteins from a sample by using LC-MS/MS-MS data

through this approach may have some error due to variations at any step reducing the reproducibility and reliability [61]. Significant improvements have been made in this technique over the last decade, such as the modifications in methods used for sample preparation, the source and amount of primary antibodies used [62, 63]. In recent years, some new protocols have also been introduced, such as DqiWest automated microfluid western blotting, capillary and microchip electrophoresis and single cell resolution [64]. So, with the help of these innovative developments in the protocol and instrumentation, sensitivity and reproducibility of western blotting can be increased [64, 65]. The principle of the western blotting is mainly based on the nature of proteins (intracellular or extracellular), quantity of specific proteins, composition of gel matrix used for proteins separation and antigen-antibody binding during the identification of specific proteins (Fig. 1).

6. PROTEIN IDENTIFICATION

6.1. Mass Spectrometry

For the identification of proteins, experimental spectra are compared with theoretical spectra obtained from protein databases [66]. Mass Spectrometry (MS) ionizes the chemical compounds into charged molecules and measures its mass to charge ratio. Although the technique was discovered in 1900s, but the scope was limited until other potential tools emerged. Samples for MALDI-TOF are prepared by coating the sample with matrix. The matrix is an organic chemical compound with an ability to absorb energy. Crystallization of matrix consequently crystalizes the protein sample (Fig. 1). A laser beam ionizes the sample coated with matrix. Upon ionization, proteins from a specific sample get protonated and separated on the basis of charge and mass ratio upon acceleration on fixed potential. These proteins are identified and measured using different mass analyzers. Time of Flight (TOF) analyzer is preferred option for microbiological uses [45].

Tandem Mass Spectrometry is one of the most used techniques for proteomics after the digestion of proteins. The advancement in the field of matrix assisted laser desorption ionization (MALDI) has increased the scope of MS's application for protein identification [67]. In MALDI-TOF technique, mass to charge ratio is calculated by determining the time it requires to travel the tube [68]. Followed by MS analysis, theoretic peptides from protein databases are checked for proximal correlation with resulting spectra (Fig. 3). The technique's efficiency has provided the potential for the use of the developed approach for identification, quantification and detection in large-scale metaproteomics [69]. After the digestion, it matches the resulting spectra with theoretical spectra of the protein database [70]. The proteomic data obtained from mass spectrometry and protein sequences are added to the Proteome

Xchange Consortium [71]. Standard proteomic softwares are often incompatible for metaproteomics search because they do not provide sufficient data on un-sequenced species and complete taxonomy of microbial communities [66].

Sequential Windowed Acquisition of all theoretical fragment ions Mass Spectrometry (SWATH-MS) is a recent development in the field of MS. Advancements in this technique gives us more accurate and reproducible results of low abundance microbial proteins. SWATH-MS uses an approach that simultaneously scans all ionized fragments in a given sample. Spectral library is used to match and identify peptide sequences with already known peptides (Fig. 1 and 3). Moreover, the abundance of peptides is quantitatively measured by extracting the targeted signals [72]. Mass Spectrometry performance varies in analyzing different sample which is one of the factors that affects the reproducibility and accuracy of proteomics results. To resolve the issue, several techniques have been developed, i.e. tandem mass tags (TMT) and isobaric tags for relative ad absolute quantification (iTRAQ). TMT and iTRAQ multiplex several samples in one analysis, reducing the quantitation error [73, 74]. A further development in mass spectrometry could yield a better throughput. Unlike genomic studies, the metaproteomics technologies that are based on mass spectrometry have the potential to provide a deeper understanding of functional interactions between host and microbes [75, 76].

6.2. SIP-Protemics (Stable Isotope Probes Linking Proteins)

Metaproteomics provides the complete information about the different proteins and enzymes to be found in a specific environment and their possible origin. To determine the function of a given enzyme from a particular environmental sample, stable isotope probes can be used. In this technique, environmental samples such as soil are labeled with isotope ^{15}N or ^{13}C to detect the functional relationships among different microorganisms [6, 77]. Microorganisms in this environment are able to incorporate ^{15}N or ^{13}C into their molecules; DNA, RNA and proteins [78, 79]. By using stable isotope probes (DNA/RNA-SIP), microbial populations can be quantified and identified directly from environmental samples.

7. SHOTGUN PROTEOMICS ANALYSIS

For shotgun proteomics analysis, two methods, (1) data-independent acquisition and (2) data-dependent selection of proteins with specific function are commonly used. These approaches can be used for comparative analysis and functional analysis of different proteins. This technique can also be used for the whole proteome analysis of various environmental samples such as blood, water and soil [80, 81]. Washburn et al. [82] have used shotgun metaproteomics for the analysis of whole proteome of yeast (*Saccharomyces cerevisiae*). They identified more than 1400 known proteins and some unknown or rarely identified proteins such as protein kinases, DNA replication and transcription factor proteins.

8. STATISTICAL ANALYSES OF METAPROTEOMES

A number of statistical software's can be used to find the correlations among diversity analysis and different environmental factors. Multivariate analyses such as principal component analysis (PCA), correspondence analysis (CA), non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) are the most common methods used for metaproteomic analyses [83, 84].

9. CONCLUSION

Metaproteomic studies of the rhizosphere soils have permitted the analysis of individual proteins that are involved in complex metabolic pathways. This approach provides a detailed study of the structure and functions of soil microbial communities together with metagenomics and transcriptomics. Thus, this review mainly focused on overview of the study of soil metaproteomics and improvements in methods for extraction, purification, and identification of soil proteins. A few protocols can be established and standardized for the extraction of soil proteins from different environments. For the separation and identification of peptides and proteins, especially proteins with small amount, specific strategies should be used. Due to lack of advanced software's and database gaps, metaproteomics technique needs some improvements to give an accurate and detailed picture of soil proteins. In future, advancement in metaproteomic techniques and

databases will be used for better understanding of functional microbial communities from different soils.

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Research Article

Growth Performance of *Labeo rohita and Gibelion catla* against Different Oilseed Meal Based Diets in Semi-intensive Culture

Javairia Shafi*, Kashifa Naghma Waheed, Muhammad Zafarullah, and Hafiza Fariha Anum

Fisheries Research and Training Institute, Lahore, Pakistan

Abstract: The present study was conducted to investigate efficacy of three different types of feeds formulated with low cost oil seed meal based proteins for growth of *Labeo rohita* and *Gibelion catla* cultured in semi-intensive system for 92 days. Formulated feeds incorporated soybean meal, guar meal and canola meal as major protein source with rice polish as control feed. Highest weight gain for *L. rohita* and *G. catla* was achieved with soybean meal based feed (121.8 g ± 4.02 g and 66.5 g ± 4.57 g) followed by canola meal based feed (107.5 g ± 17.1 g and 47.3 g ± 8.59 g), guar meal based feed (49.4 g ± 10.3 g and 19.6 g ± 8.55 g) and rice polish (31.8 g ± 1.40 g and 14.1 g ± 3.06 g), respectively. Growth performance of *L. rohita* was higher than *G. catla* in all treatments. During the experimental period, net production (kg/ tank) was 1.59 kg ± 0.07 kg, 6.02 kg ± 0.20 kg, 2.48 kg ± 0.52 kg and 5.37 kg ± 0.85 kg for *L. rohita* and 0.71 kg ± 0.15 kg, 3.32 kg ± 0.23 kg, 0.98 kg ± 0.43 kg and 2.37 kg ± 0.43 kg for *G. catla* achieved with rice polish, soybean meal, guarl meal and canola meal based feeds respectively. Results showed that soybean meal and canola meal based formulated diets can lead to higher fish growth and total fish production than other oilseed meals used in present investigation.

Keywords: Aquaculture, Formulated feed, Growth performance, Major carps, Condition factor.

1. INTRODUCTION

World population growth rate demands an increased food supply and sustainability of current food production systems [1]. Aquaculture is growing at the fastest rate among world's food production sectors [2] and can serve as a valuable resource to fight against the global issues of malnutrition and poverty. Fish and fish products are the source of highly digestible proteins in human diets comprising of all the essential amino acids [3]. Due to its high quality protein and other nutritional qualities, global fish per capita consumption has increased from 9.9 kg in 1963 to 19.7 kg in 2013 [4]. At present, the challenges faced by the sustainable growth of aquaculture are directly related to nutrition and feeding requirements of cultured fish [5, 6].

Fish oil and fish meal, the two major biological sources required to fulfill the feed based requirements of sector, are derived from world's declining marine capture fisheries resources [7]. In 2006, about 88.5% of total fish oil production in the world and 68.2% of world's total fish meal production was used for aquaculture feed inputs [8]. This reliance on finite natural fisheries resources not only lead to increased prices of pelagic fish used as fish meal but also result in increased market rates of cultured fish species as compared to their captured counterparts [9]. Therefore, the continuous development of aquaculture necessitates availability of economically compatible aqua feeds to ensure sector's viability for satisfying the protein demand of growing global population.

Efforts carried out to develop cost effective feeds for finfish are directed towards partial or complete replacement of fish meal with compatible raw materials of animal or plant origin. Oil seed meals; the byproducts leftover after oil extraction from oil bearing seeds are the most important plant based ingredients for use in aqua feeds due to their high protein (20-50%) content [10]. They are the focus of worldwide research in recent years for

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* Corresponding Author: Javairia Shafi; javairiamalik@gmail.com

preparation of low cost diets for freshwater finfish and have resulted in promising results [11-13].

In Pakistan, freshwater aquaculture practices have been concentrated towards polyculture of major carps under semi intensive systems. Fish nutrition is considered an active area of research in the region for the development of economically compatible feeds that can aid the fish farmers with limited resources. None of the earlier investigations has compared oilseed meal based aquafeeds in culture of more than one species in semi intensive system. Present study was, therefore, carried out to evaluate growth of *Labeo rohita* (Rohu) and *Gibelion catla* (Catla) fed with plant based formulated feeds to supplement the research carried out on formulation of low cost feed for carp's culture.

2. MATERIALS AND METHODS

2.1 Study Site

The experiment was conducted for 92 days starting from 9 June, 2017 to 8 September, 2017 in outdoor cemented tanks at Fisheries Research and Training Institute (FR&TI), Lahore, Pakistan. The experiment was conducted in outdoor cemented cisterns each with dimensions of 8.9 m × 2.18 m × 1.16 m (length x width x depth). The bottom of tanks was covered with 0.015 m layer of soil and tanks were filled with water up to 0.85 m. Water volume in each tank was maintained at 53.36 m^3. Dissolved oxygen level in all the tanks was maintained through coarse bubble aeration. Any kind of organic/ inorganic fertilizers was not applied in treatment tanks.

2.2 Feed Formulation

Low cost, readily available raw materials were used to formulate three different types of feeds based on soybean meal, guar meal or canola meal (Table 1). Rice polish (by product of rice milling process) was used as control. Each type of feed was prepared by mixing the ingredients in required proportion followed by their grinding and homogenization in a feed mill. To determine the nutritional characteristics of prepared feeds, moisture content (105 °C, 2 hours), ash content (600 °C, 2 hours), crude protein (Kjeldahl digestion) and fat content (Soxhlet extraction) of feeds and their raw materials was determined in triplicate by Standard Methods as described in AOAC [14]. For first 23 days of experimental period, fry in each tank were fed with experimental diets in quantity equal to their total fish body weight stocked in each tank. Later, as fish grew in size and reached the fingerling stage, experimental diets were supplied at 5% of total fish body weight. In either case, feed in equal doses was supplied twice a day at fixed time intervals.

2.3 Fish Species and Stocking Density

Fry of two major carps, *L. rohita* and *G. catla* were procured from Fish Seed Hatchery, Mian Channu, Pakistan. Fish seed was transported to FR & TI, Lahore in plastic bags filled with appropriate amount of water and saturated with oxygen. Fish fry were acclimatized in separate cemented tanks for one week and fed with rice polish at weight equal to total body weight of fish stocked in each tank. At the end of acclimatization period, fry of each

Table 1. Composition of the experimental feeds

Feed Ingredients	Experimental Feeds			
	T1 (Control) (%)	T2 (%)	T3 (%)	T4 (%)
Rice polish	100	-	-	-
Soybean meal	-	70	-	-
Guar meal	-	-	70	
Canola meal	-	-		70
Wheat flour	-	15	15	15
Corn glutton meal	-	5.0	5.0	5.0
Rice bran	-	8.9	8.9	8.9
Vitamin & Mineral premix	-	1.0	1.0	1.0
Table salt	-	0.10	0.10	0.10
Total	100	100	100	100

major carp were randomly stocked in cemented tanks after recording of their average body weight and total length. Two major carps were stocked in stocking ratio of 1:1 and total stocking density of six fry in one cubic meter (6 fry/m^3).

2.4 Growth Parameters

Five specimen of each fish species were randomly captured from each tank after each 23 days for growth monitoring in terms of body weight and total length. Feed was continuously adjusted according to increase in fish biomass in each tank throughout the research period. At the end of experimental period, all fish specimens from each experimental tank were harvested for recording of fish survival, final wet body weight and total length. The collected data was used to calculate fish weight gain (WG), average daily weight gain (ADWG), length gain (LG), average daily length gain (ADLG), specific growth rate (SGR), survival rate (SR), condition factor (CF), gross & net production and feed conversion ratio (FCR) for each fish species by application of suitable formulae. Condition factor was calculated according to Froese [15] using the following formula:

Condition Factor: (W/L^3) x 100

Where W: fish weight; L: Fish length

2.5 Water Quality Monitoring

The physico-chemical parameters i.e., water temperature, dissolved oxygen, pH and conductivity were monitored daily while total alkalinity, chloride content, total hardness and calcium hardness were analyzed on monthly basis. Water temperature was measured using glass thermometer while pH and conductivity were determined using pH meter (Jenway, 3505) and conductivity meter (Jenco, 3173), respectively. Total dissolved solids were calculated by multiplying water conductivity with a factor of 0.85. Dissolved oxygen was measured by Winkler method with azide modification [16]. Total alkalinity, total hardness, calcium hardness and chloride content were determined by volumetric titrations using standard methods as described in APHA [16]. A schematic presentation of methodology has been presented in Fig. 1.

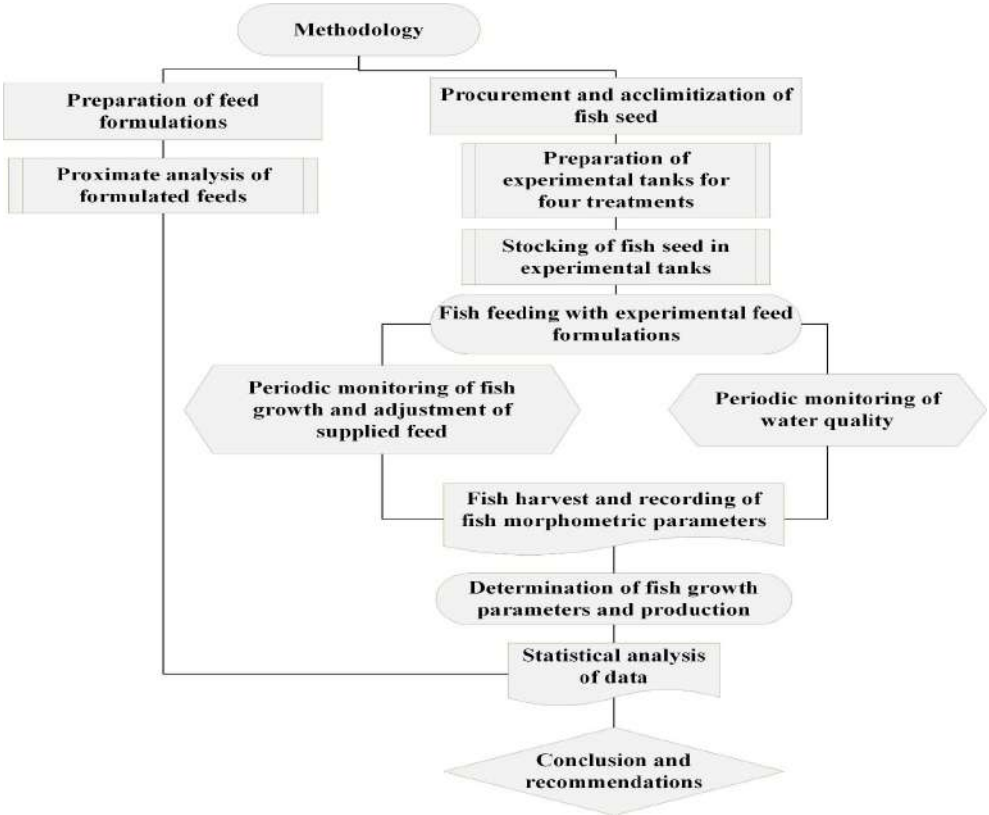

Fig 1. Schematic representation of methodology

2.6 Statistical Analysis

Statistical analysis of the data was carried out through SPSS (ver. 16.0) to find out statistically significant differences in growth performance of cultured fish species under various treatments through one way analysis of variance at $P<0.05$. Post hoc analysis was carried out to find differences between pair of means by Fisher's Least Significant Difference (LSD) test.

3. RESULTS

Proximate composition of experimental feeds and their ingredient has been presented in Table 2. Soybean meal based feed (T2) contained highest crude protein content (31.32% ± 0.32%) followed by guar meal based feed (T3) (26.20% ± 1.92%)) and canola meal based feed (T4) (24.54% ± 2.97%). Rice polish used as control diet in T1 was found to have lowest crude protein (12.19% ± 0.48%). However, its ash and crude fat content was higher as compared to formulated feeds used in rest of treatments.

Growth performance of Rohu and Catla has been presented in Table 3 along with gross and net production. Survival rate (SR) for both species was found to be 100% in all treatments. Analysis of variance indicated significant differences in growth of two fish species achieved in various feed treatments at $P< 0.05$. For Rohu, weight gain achieved in T2 and T4 was significantly higher than that of T3 and T1. However, for Catla, weigh gain in T2 was significantly higher than other three treatments. Weight gain decreased in the following order for four experimental feeds. Soybean meal based feed > Canola meal based feed > Guar meal based feed > Rice polish based feed.

For Rohu, highest WG was achieved in T2 (121.85 g ± 4.02 g) followed by T4 (107.48 g ± 17.07 g) and T3 (49.45 g ± 10.30 g). Control treatment (T1) showed least WG (31.77 g ± 1.40 g) for Rohu. Highest weight gain of Catla was found in T2 (66.47 g ± 4.57 g) followed by T4 (47.34 g ± 8.59 g), T3 (19.63 g ± 8.55 g) and T1 (14.14 g ± 3.06 g). In case of Rohu, SGR was highest in T4 followed by T2, T3 and T1 respectively. However, for Catla, SGR was highest in T2 followed by T4, T3 and T1 respectively. Differences between WG, ADWG and SGR of Rohu and Catla as achieved in T2 and T4 were not significant ($P<0.05$). Increase in weight of Rohu and Catla under all treatments over the entire experimental period has been shown in Fig. 2 and Fig. 3 respectively.

Net production (kg/tank) of both fish species was higher in T2 and T4 than that of T1 and T3. Lowest FCR (1.93 ± 0.06) was found in T4 for Rohu and in T2 (1.67 ± 0.15) for Catla. Control diet (T1) showed highest FCR for both fish species i.e. Rohu and Catla (2.73 ± 0.59 and 2.35 ± 0.41), respectively. Condition factor (CF) varied from

Table 2. Proximate composition (Mean ± Standard Deviation (SD)) of feed ingredients and experimental feeds

Feed Ingredient/ Formulated Feed	Proximate Composition			
	Moisture (%)	Ash (%)	Crude Protein (%)	Crude Fat (%)
Soybean meal	10.4 ± 1.94	6.59 ± 0.04	39.3 ± 1.67	1.12 ± 0.51
Guar meal	7.50 ± 0.24	4.61 ± 0.03	32.0 ± 2.43	4.80 ± 0.25
Canola meal	9.27 ± 0.56	6.78 ± 0.30	29.6 ± 0.48	4.29 ± 1.77
Wheat flour	9.39 ± 0.34	1.64 ± 0.06	11.4 ± 2.33	3.21 ± 0.41
Corn gluten meal	10.7 ± 0.23	8.35 ± 0.21	16.6 ± 0.49	1.93 ± 0.04
Rice bran	11.5 ± 0.17	0.53 ± 0.04	9.99 ± 0.18	0.33 ± 0.18
Feed in T1 (rice polish)	8.95 ± 0.30	9.26 ± 0.02	12.2 ± 0.48	15.2 ± 1.20
Formulated feed in T2 (based on soybean meal)	9.19 ± 0.19	5.72 ± 0.03	31.3 ± 0.28	1.02 ± 0.20
Formulated feed in T3 (based on guar meal)	7.94 ± 0.05	4.66 ± 0.03	26.2 ± 1.92	3.47 ± 0.68
Formulated feed in T4 (based on canola meal)	8.44 ± 0.45	6.23 ± 0.08	24.5 ± 2.97	1.94 ± 0.61

1.43 ± 0.05 to 1.27 ± 0.0 for Rohu and from 1.45 ± 0.06 to 1.33 ± 0.0 for Catla. In case of Rohu, highest CF was found in T4 followed by T2, T3 and T1 respectively. For Catla, highest CF was found in T2 followed by T4, T3 and T1. The physico-chemical parameters of water were found to be within suitable ranges as described by Boyd and Tucker [17] for cultured fish species and are presented in Table 4.

Table 3. Growth performance (Mean ± SD) of Rohu and Catla under different feeding treatments

Parameters	Fish name	Treatments			
		T1	T2	T3	T4
Initial weight (g)	Rohu	0.57 ± 0.16	0.46 ± 0.03	0.47 ± 0.01	0.32 ± 0.02
	Catla	0.18 ± 0.04	0.11 ± 0.00	0.17 ± 0.08	0.10 ±0.00
Final weight (g)	Rohu	32.3 ± 1.60^{b}	122.3 ± 4.04^{a}	49.9 ± 10.3^{b}	107.8 ± 17.0^{a}
	Catla	14.3 ± 3.03^{c}	51.5 ± 4.57^{a}	19.8 ± 8.63^{c}	47.4 ± 8.58^{b}
Weight gain (g)	Rohu	31.8 ± 1.40^{b}	121.8 ± 4.02^{a}	49.4 ± 10.3^{b}	107.5 ± 17.07^{a}
	Catla	14.1 ± 3.06^{c}	66.5 ± 4.57^{a}	19.6 ± 8.55^{c}	47.34 ± 8.59^{b}
Average daily weight gain (g)	Rohu	0.35 ± 0.02^{b}	1.32 ± 0.04^{a}	0.54 ± 0.11^{b}	1.17 ± 0.18^{a}
	Catla	0.16 ± 0.03^{c}	0.74 ± 0.05^{a}	0.22 ± 0.09^{c}	0.53 ± 0.10^{b}
Specific growth rate (%)	Rohu	4.41 ± 0.25^{c}	6.07 ± 0.03^{a}	5.06 ± 0.19^{b}	6.30 ± 0.24^{a}
	Catla	4.89 ± 0.46^{b}	7.13 ± 0.07^{a}	5.30 ± 0.08^{b}	6.76 ± 0.25^{a}
Length gain (cm)	Rohu	10.5 ± 0.71^{b}	17.1 ± 021^{a}	13.2 ± 1.70^{b}	17.0 ± 1.20^{a}
	Catla	8.68 ± 0.32^{b}	15.0 ± 0.60^{a}	9.80 ± 0.99^{b}	13.7 ± 0.33^{a}
Average daily length gain (cm)	Rohu	0.12 ± 0.01^{b}	0.19 ± 0.00^{a}	0.15 ± 0.02^{b}	0.19 ± 0.01^{a}
	Catla	0.10 ± 0.00^{b}	0.17 ± 0.00^{a}	0.11 ± 0.01^{b}	0.15 ± 0.00^{a}
Condition factor	Rohu	1.27 ± 0.00^{b}	1.42 ± 0.07^{a}	1.34 ± 0.01^{a,b}	1.43 ± 0.05^{a}
	Catla	1.33 ± 0.00^{b}	1.45 ± 0.06^{a,b}	1.35 ± 0.04^{a,b}	1.39 ± 0.01^{a,b}
Survival rate (%)	Rohu	100.0	100.0	100.0	100.0
	Catla	100.0	100.0	100.0	100.0
Gross production (kg.tank^{-1})**	Rohu	1.62 ± 0.08^{b}	6.12 ± 0.20^{a}	2.50 ± 0 .52^{b}	5.39 ± 0.85^{a}
	Catla	0.72 ± 0.15^{b}	3.33 ± 0.23^{a}	0.99 ± 0.43^{b}	2.37 ± 0.43^{a}
Gross production** (kg.ha^{-1})	Rohu	808 ± 39^{b}	3058 ± 101^{a}	1248 ± 258^{b}	2695 ± 426^{a}
	Catla	358 ± 76^{b}	1664 ± 114^{a}	495 ± 216^{b}	1186 ± 215^{a}
Net production (kg.tank^{-1})**	Rohu	1.59 ± 0.07^{b}	6.09 ± 0.20^{a}	2.48 ± 0.52^{b}	5.37 ± 0.85^{a}
	Catla	0.71 ± 0.15^{c}	3.32 ± 0.23^{a}	0.98 ± 0.43^{c}	2.37 ± 0.43^{b}
Net production** (kg.ha^{-1})	Rohu	794 ± 35^{b}	3046 ± 100^{a}	1236 ± 258^{b}	2687 ± 426^{a}
	Catla	353 ± 76^{c}	1662 ± 114^{a}	491 ± 214^{c}	1184 ± 215^{b}
FCR	Rohu	2.73 ± 0.59^{a}	1.98 ± 0.14^{a}	1.94 ± 0.34^{a}	1.93 ± 0.06^{a}
	Catla	2.35 ± 0.41^{a}	1.67 ± 0.15^{b}	1.80 ± 0.18^{a,b}	1.89 ± 0.04^{a,b}

*: Means that do not share a letter in a same row are statistically significant (P<0.05).
**: During 92 days experimental period

Table 4. Water quality parameters (Mean ± SD) recorded during experimental period

Parameter	Treatments			
	T1	T2	T3	T4
Temperature (°C)	30.7 ± 0.03	30.8 ± 0.00	30.84 ± 0.01	30.7 ± 0.09
Dissolved oxygen (mg.L^{-1})	5.60 ± 0.37	5.40 ± 0.00	5.80 ± 0.11	5.60 ± 0.36
pH	8.50 ± 0.00	8.52 ± 0.03	8.57 ± 0.01	8.53 ± 0.01
Conductivity (µS.cm^{-1})	579.3 ± 4.92	589.0 ± 5.76	575.2 ± 1.66	593.9 ± 4.31
Total dissolved solids	10.7 ± 0.23	8.35 ± 0.21	16.6 ± 0.49	1.93 ± 0.04
(mg.L^{-1})	492.4± 4.18	500.6± 4.90	488.8 ± 1.41	504.8 ± 3.66
Total alkalinity (mg.L^{-1})	367.6 ± 3.52	366.4 ± 7.03	364.2± 1.40	356.5 ± 9.38
Chloride (mg.L^{-1})	14.1 ± 0.16	16.3 ± 0.55	14.9 ± 0.80	15.6 ± 0.00
Total hardness (mg.L^{-1})	171.8± 10.6	153.3 ± 4.84	161.9 ± 1.10	162.0 ± 6.05
Calcium hardness (mg.L^{-1})	56.6 ± 1.83	58.6 ± 7.28	61.4 ± 0.18	75.9 ± 0.82

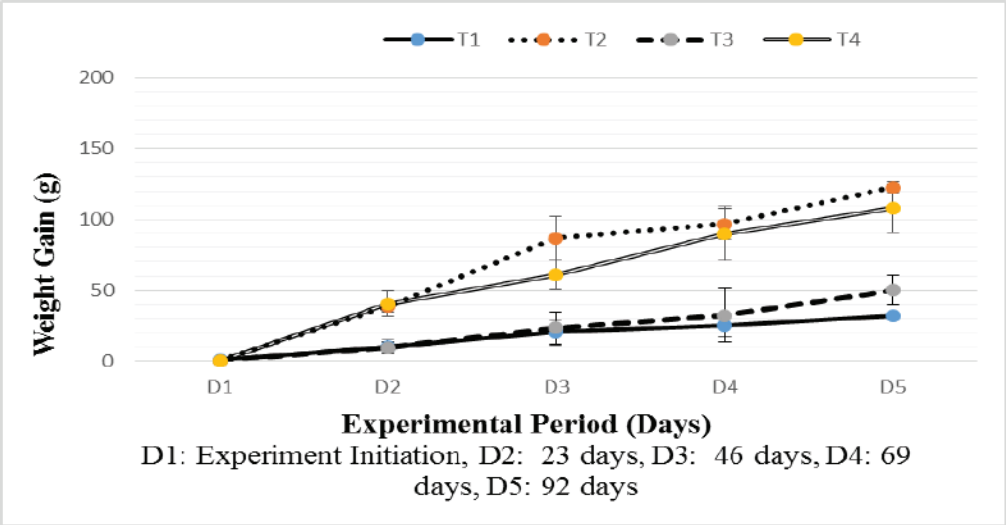

Fig. 2. Weight gain (g) of Rohu during experimental period (Error bars show SD)

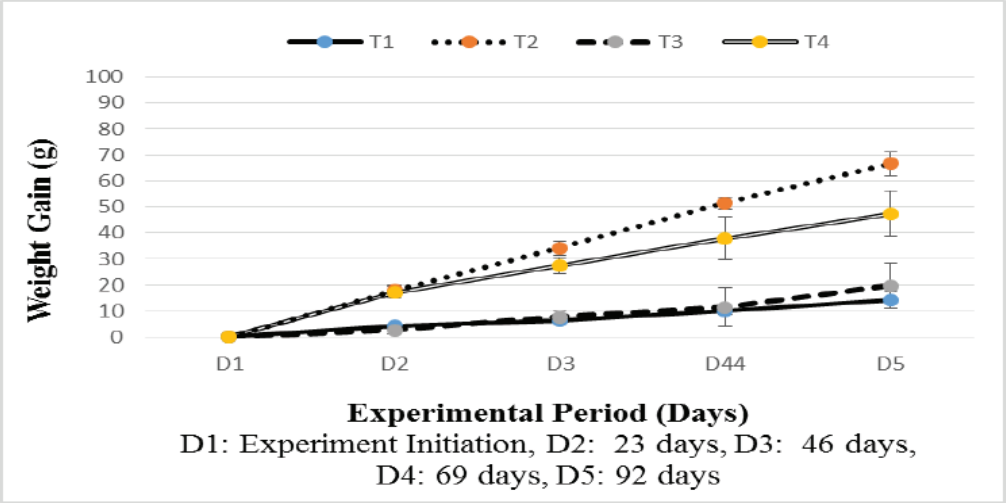

Fig. 3. Weight gain (g) of Catla during experimental period (Error bars show SD)

4. DISCUSSION

Optimum fish growth and health which is the primary objective of aquaculture activities depends upon appropriate nutrition of cultured animals. Exogenous feeds providing minerals, vitamins and essential nutrients are the crucial source of nutrition in intensive and semi intensive fish culture. Raw materials used in fish feed formulations should be digestible, palatable and of high nutritive quality. Historically, fish meal has been used as major protein source in aqua feeds due to its nutritional characteristics and digestibility [18]. However, on the basis of current rate of fish meal consumption in aquafeeds and expected development of aquaculture in future, it has been estimated that sector's demand for fishmeal will outrun the annual production of latter [19]. Use of fishmeal based aquafeeds not only raise concerns about viable development of aquaculture but also result in an increase in challenging issues that the sector has to cope with in future. Use of trash fish in feed that can lead to viral and bacterial infection of cultured fish [20] adulteration of fish feeds with melamine to artificially increase its protein content [21], use of contaminated fish meal in feed preparation and subsequent bioaccumulation of toxic materials in cultured animals [22] are to name a few. This scenario has incentivized the replacement of fish meal with other readily available, cost effective alternatives of high nutritional quality. The only solution to this dilemma is replacement of fish meal with low cost and readily available animal and plant based raw materials without any compromise on growth and production of cultured animals. Fortunately, low trophic level finfish are more acquiescent in terms of protein source in their feed and not necessarily require fish meal and fish oil based diets like carnivorous species and shellfish [23].

In present study, efficacy of four different types of formulated feeds based on cost effective ingredients from plant sources were evaluated in terms of growth performance of two major carps. A comparison of crude protein content of plant feed stuff as determined in the present study with the values cited in literature has been described in Table 5. Plant based ingredients are deficient in certain amino acids whose adequate supply in feed is imperative for fish [24]. Soybean meal, for example, contain limited concentration of sulphur bearing indispensable amino acid; methionine [25]. This obstacle can, however, be overcome by either supplementation of plant based feedstuff with limiting amino acids [26] or alternatively formulation of the feed with plant feedstuffs of different origin that can supplement amino acids deficiency of each other [27, 28]. In the present study, second strategy was adopted and corn gluten meal, wheat flour and rice bran were used to reduce amino acid deficiency caused by limiting amino acids of basic oilseed meals used. Nutritional composition of formulated feeds was balanced by addition of vitamins and mineral premix and table salt. Highest fish growth in terms of weight gain and average daily weight gain of Rohu and Catla was achieved with Soybean meal based formulated feed. The results are in agreement with those of Khan *et al*. [26] who reported higher weight gain with

Table 5. Comparison of crude protein content of feed ingredients with literature

Feed ingredient	Crude protein content (%) (Literature based value)	Crude protein content (%) (Present study)
Rice polish	4.70-14.9[30]	12.2
Soybean meal	49.2[31]	39.3
Guar meal	33.0 - 45.0[32]	32.0
Canola meal	36.5[31]	29.6
Wheat flour	7.13 -14.4[33]	11.4
Corn gluten meal	60.0[34]	24.7*
Rice bran	12.5[35]	9.99

*Commercial corn gluten meal (30%) that claims to be 30% protein content and is prepared by mixing of corn gluten meal with corn gluten feed to produce low protein content variet

soybean meal based diets than those of groundnut and canola meal based diets and concluded that soybean meal supplemented with methionine and minerals can effectively replace fish meal in Rohu feed. Rehman *et al*. [29] have also reported that fish meal can be partially replaced with soybean meal and sunflower meal in diets of Rohu without any negative effect on fish growth.

Results of present study are contradictory with those Iqbal *et al.* [36] who reported highest weigh gain of Rohu with guar meal than canola meal, soybean meal, cottonseed meal and fish meal. Lowest weight gain of both cultured fish species with rice polish are in agreement with those of Abid and Ahmed [37] and Ahmed *et al.* [38] who have also reported least weight gain of Rohu with rice polish when compared with other plant materials based diets. Higher FCR values found in present study are in line with those of Khan *et al.* [26] who have reported FCR of 2.2 ± 0.06 for Rohu fed with plants based diet based on soybean meal. Growth performance of Rohu was higher than Catla in terms of WG, ADWG, LG, ADLG and production in present investigation. SGR of Catla was, however, found to be higher than Rohu under all treatments. Condition factor has been considered as an indicator of fish health as higher condition factor relates to healthier fish [39]. Higher condition factor of Rohu and Catla fed with soybean meal based feed and canola meal based feed indicated improved fish health in these treatments.

According to present study, use of feeds formulated with soybean meal and canola meal as source of oilseed meal has resulted in higher fish production and weight gain. Net production of Rohu ($kg.ha^{-1}$) achieved with soybean meal based diet was about 59% and 74% higher than that of guar meal and rice polish based diets respectively. In the case of Catla, net fish production ($kg.ha^{-1}$) with soybean meal based feed was 70% and 79% higher than that found with guar meal and rice polish based diets respectively. For canola meal based feed, net Rohu production was found to be 54% & 70% and net Catla production was 58% and 70% higher than that of guar meal and rice polish based diets respectively. A comparison of estimated costs of formulated feeds and commercial feeds available in market for carps has been presented in Table 6. Estimated cost of soybean meal based feed is higher than that of rest of the experimental diets due to relatively high rate of soybean meal. However, the rates of soybean meal based feed and canola meal based feed are about 26% lower than that of commercially available feed of comparable protein content (CP; 30% and 23% respectively). Higher growth of fish fed with formulated diet containing soybean meal can be attributed to its amino acid composition that is considered as one of the best among plant based raw materials to meet the fish demand for indispensable amino acids [10]. Soybean meal is often referred as "gold standard" in aquafeeds due to its high nutritional value and abundant availability in international market feed raw materials [40].

5. CONCLUSION

Present study has led to the conclusion that soybean meal complemented with corn gluten meal, rice bran and wheat flour can be used in formulation of cost effective diets for *L. rohita* and *G. catla*. In future, there is the need to evaluate effects of long term use of plant based aquafeeds on fish physiology, nutritional quality and consumer health.

Table 6. Comparison of cost of formulated feeds with available commercial feed

Feed	CP (%)	Estimated Cost		Net production ($kg.ha^{-1}$)	
		(PKR*/kg)	(PKR*/50kg)	*L. rohita*	*G. catla*
Feed in T1 (rice polish)	12.2	23.0	1150.0	1.59	0.71
Formulated feed in T2 (based on soybean meal)	31.3	63.0	3135.0	6.09	3.32
Formulated feed in T3 (based on guar meal)	26.2	44.0	2190.0	2.48	0.98
Formulated feed in T4 (based on canola meal)	24.5	47.0	2365.0	5.37	2.37
Commercial feed (available in market)	30.0	85.0	4250.0	---	
Commercial feed (available in market)	23.0	64.0	3187.0	---	

*PKR: Pakistani Rupees

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Research Article

Quality of Tomatoes as Influenced by Bio-Chemicals and Controlled Atmosphere during Storage

Shahid Masood[1], Muhammad Atif Randhawa[1], Waqas Ahmad[2*], Masood Sadiq Butt[1], Muhammad Asghar[1], and Saqib Jabbar[3]

[1]National Institute of Food Science and Technology, University of Agriculture, Faisalabad
[2]Horticulture lab College of Agriculture BZU, Bahadur Sub-Campus Layyah
[3]Food Science and Product Development Institute, National Agricultural Research Centre, Islamabad

Abstract: A study was conducted to assess the potential of neem leaf extract, *Aloe vera* gel and Chitosan on the quality of two tomato cultivars (Sahil& Rio Grande) during storage in comparison with traditional post-harvest fungicide thiophanate methyl. Total soluble solids, acidity, color, respiration rate and ethylene production of bio-pesticides, treated tomatoes were compared (at 7 day interval, till 35 days) with those of treated tomatoes (thiophanate methyl) and control sample. Treated tomatoes (both varieties) were stored at 10°C and 75% humidity without CO_2 and with 3% CO_2 for 35 days in two different environment chambers. The selected parameters which affect the post-harvest quality loss were significantly controlled in the treated tomatoes (neem leaf extract and stored with 3% CO_2) as compared to control and all other treatments except thiophanate methyl that also showed a significant effect.

Keywords: Bio-chemicals, Controlled atmosphere, Storage life, Tomatoes

1 INTRODUCTION

Maintaining food quality and ensuring the food safety are two major objectives of storages and packing houses. Selecting the best options and integrating the various components in a safe and cost-effective management scheme has added complexity to storage and packing operations. In addition, there is increasing scrutiny of postharvest practices for all fresh produce due to the hazardous pesticide residual effects. Socioeconomic and environmental damage due to application of hazardous pesticides on crop plants is estimated about 8.1billlions dollars annually [1].

Biopesticides are important areas to fulfill the challenges in a sustainable way. Biopesticides are derived from natural materials such as animals, plants, bacteria, and certain minerals widely used for controlling insects and disease causing pathogens by non-toxic mechanisms. Demand for chemicals free crop products is expected to enhance the demand for biopesticides in near future. Organic food market are other driving factors for increasing trend in biopesticides market, since future organic industry is solely dependent upon the chemical free crop protection products to safeguard the environment. The global biopesticide market was valued at 1.3 billion dollars in 2011 and is expected to expand much more in nearby future.

Europe is expected to be the fastest growing market in the near future owing to the strict laws for pesticides. Biopesticides are usually inherently less toxic; generally affect only the selective pest, effective in very small quantities, easily biodegradable, thereby resulting in lower exposures and largely avoiding the environmental pollution. When used as a component of Integrated Pest Management programs, it can greatly control major pest threat without affecting the crop yield [2]. As of early 2013 there were approximately 400

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* Corresponding Author: Waqas Ahmad; uaf_waqas@yahoo.com

registered active ingredients of biopesticides and more than 1250 actively registered biopesticide products [3].

Herbal extracts have gained a lot of attention because of numerous potential applications and properties. Antibacterial, antifungal and film forming properties of plant extracts has made their use an ideal to improve the stability of perishable fruits. A large number of chemicals have been developed for the control of postharvest diseases of crop plants. But due to hazardous side effects of these chemicals, more and more emphasis is being given to the use of bio control agents. Now major challenge is felt in the field of plant pathology to introduce some eco-friendly and safe alternative control strategies for agricultural commodities, which led researchers to turn their attention to herbs and plants as sources of bio agents to control postharvest decay of farm products during storage. Recently, our team has been published research on the potential of biopesticides to enhance the shelf life of tomatoes [4] and the present study was the further extension of our research work on different quality parameters.

2. MATERIALS AND METHODS

2.1. Chemicals

Sodium hydroxide was purchased from Fisher Scientific, UK. Methanol was procured from VWR International, Geldenaaksebaan 464, 3001, Belgium. Sodium hypochlorite, glacial acetic acid, phenolphthalein, citric, chitosan, pectin and ascorbic acid were purchased from Sigma Aldrich Chemical Co. St. Louis, MO, USA. Topsin-M 70 WP (Thiophanate Methyl) was procured from Nippon Soda Co., Ltd. Japan.

2.2. Plant Material

Two tomato varieties (Sahil & Rio Grande) were purchased from Faisalabad (Agricultural Farm), Pakistan, according to the USDA standard tomato color classification chart [5].The mature and healthy fruits were selected. The solution of sodium hypochlorite (0.05g/100 mL) was used to wash the tomatoes and then air-dried at ambient temperature before further treatment.

2.3. *Aloe vera* Gel Preparation

Aloe vera plant (matured leaves) was obtained from Faisalabad, (Agricultural Farm) Pakistan. The sodium hypochlorite solution (0.05g/100 mL) was used to wash *Aloe vera* leaves. *Aloe vera* gel was collected from the leaves (outer cortex) and blended in a high speed blender. To remove the fibers, the resulting mixture was filtered. The obtained gel matrix after filtration was then pasteurized for 45 minutes at 70°C. The cooling of gel was carried out immediately at ambient temperature. To maintain the pH at 4, the citric acid (4.5-4.6 g/L) and ascorbic acid (1.9-2.0 g/L) were added. The pectin (1g/100 mL) was used to improve the viscosity and coating efficiency of the stabilized *Aloe vera* gel. To prevent oxidation of the gel, the glass bottle (brown color) was used to store the gel [4, 6].

2.4. Neem Leaf Extract Preparation

Neem leaf extract preparation was done by the described methodof Masood et al [4]; Subapriya and Nagini [7] with slight modification. Mature fresh leaves *of Azadirachta indica* were harvested from Faisalabad (University of Agriculture), Pakistan. After washing with sterilized distilled water, the leaves (500g) were soaked in 1000 mL absolute methanol in the flask and left for 24 hours to allow for extraction. And then coarse residues were filtered through muslin cloth. Coarse residues were extracted repeatedly with 500 mL methanol. All extracts were mixed together and coarse filter paper was used to filter them. Rotary evaporator was used to remove the methanol from the extract till the volume was about 500 mL. The air tight amber colored glass bottle was used to store the extract.

2.5. Chitosan Coating Preparation

Chitosan (30g) was dissolved in 3 liters distilled water.The distilled water contained glacial acetic acid (150 mL). The solution of NaOH (1g/L) was used to adjust pH at 5.0 [4].

2.6. Thiophanate Methyl Solution Preparation

The thiophanate methyl solution was prepared by using 1g Topsin-M 70WP (Thiophanate Methyl 70%) into 1 liter of distilled water [4].

2.7. Experiment Plan and Storage

Aloe vera gel (50g/100 mL), Chitosan (3g/100 mL), neem leaf extract (20g/100 mL) and fungicide thiophanate methyl (0.1g/100 mL) coating solutions were used for tomatoes. The fresh fruits were dipped completely into the coating solutions for 15 minutes at 30°C temperatures. The coated fruits were dried before storage. The untreated and treated tomatoes were keptat 10°C and 75% relative humidity for 35 days under controlled atmosphere (without CO_2 and with 3% CO_2). Physical and chemical analysis was done after every 7 day interval. The different treatments scheme was as: TC: tomatoes were treated with Chitosan, TN: tomatoes were treated with neem leaf extract, TA: tomatoes were treated with *Aloe Vera* gel, TF: tomatoes were treated with conventional fungicides thiophanate methyl, control: untreated tomatoes.

2.8. Respiration Rate

The rate of CO_2 production was determined by digital CO_2 meter (Inspect Air CO_2 Meter, Model 8560, TSI Incorporated, USA) as determined by Ullah et al [8].

2.9. Ethylene (C_2H_4) Production

Ethylene gas production was determined by digital meter (Drager Safety Pac III, Mexico) as determined by Ullah et al[8].

2.10. Fruit Color

Surface Color was evaluated by a colorimeter (Model Color Tec PCM+ ColorTec Associates, Inc. USA). It was expressed as the ratio between a* and b* parameter which is an indication of color transformation of turning green to red. Ratio $a^*/b^* < 0$ indicates the green color while $a^*/b^* > 0$ indicates red color [9].

2.11. Total Soluble Solid Contents

Total soluble solid contents of freshly extracted juice by using a refractometer (Model RX 5000 Atago, Japan) at 20°C temperatures were determined as per the method described by Dabeka and McKenzie [10].

2.12. Titratable Acidity

According to the standard technique of Dabeka and McKenzie, [10] acidity of juice was evaluated by titration of juice against NAOH (0.1g/L) using Titratable acidity mini titrator(Model no. HI 84432, HANNA Instruments Inc. Rumania).

2.13. Statistical Analysis

The Complete Randomized Design (CRD) with three-factor factorial was used to analyze data. The level of significance $P < 0.05$ was used. Statistix 9.0 software (Analytical Software, Tallahassee, FL, USA) was used for statistical analyses.

3. RESULTS AND DISCUSSION

3.1. Effects of Post-Harvest Bio-Chemicals on the Total Soluble Solid Contents of Tomatoes during Storage

To evaluate the fruit ripening, the total soluble solid contents are considered basic criteria. The results showed that the total soluble solid contents were very low at the time of harvest, but total sugars increased with the passage of time during ripening (Figure 1). However, during storage of tomatoes, sugar contents significantly changed in neem leaf treated tomatoes with 3% CO_2 as compared to control, Chitosan and *Aloe vera* treated tomatoes except tomatoes treated with thiophanate methyl. Changes in total soluble solid contents of thiophanate treated tomatoes were almost similar with neem leaf treated tomatoes. Effect of biochemical treatment was found non-significant with respect to varieties. Sahil and Rio grande showed the same results regarding change in total soluble solid contents during storage with and without CO_2 storage. Total soluble solid contents of neem leaf were increased from 3.63% to 4.23 with CO_2 while without CO_2 total soluble solid contents changed from 3.64 to 5.1% during 35 days of storage while total soluble solid contents of control at initial was 3.62 % and at the end of storage soluble solid contents were 5.37% with the CO_2 storage condition. While total soluble solid contents of control without CO_2 increased from 3.58 to 5.2%. Results showed that change in the total soluble solid contents of neem leaf treated tomatoes were slow during storage as compared to control and other treatments with CO_2

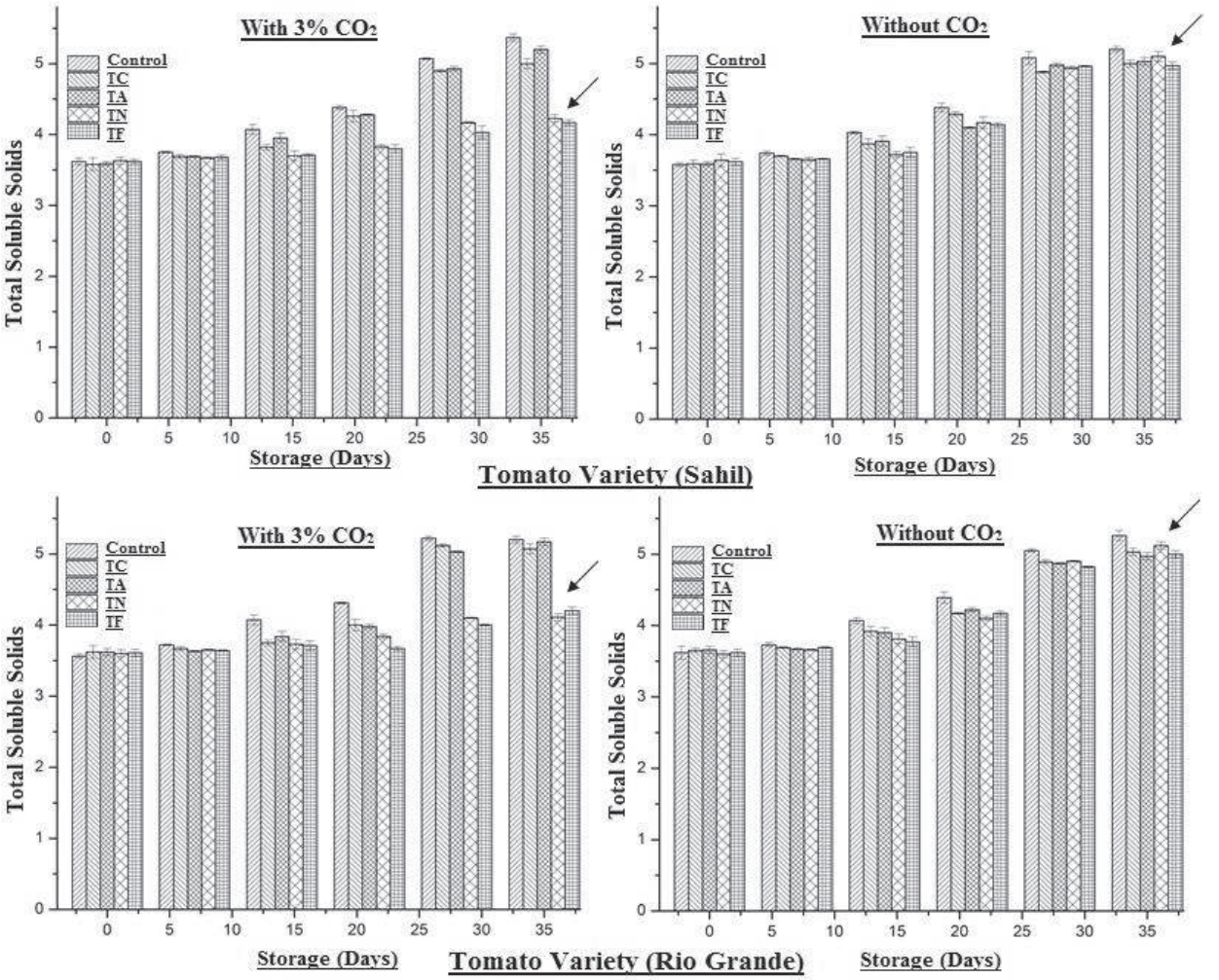

Fig. 1. Effects of post harvest treatments on the total soluble solid contents of the tomatoes during storage. Control: untreated tomatoes, TC: Tomatoes were treated with Chitosan, TA: Tomatoes were treated with Aloevera gel, TN: Tomatoes were treated with Neem leaf extract, TF: Tomatoes were treated with conventional fungicides thiophanate methyl.

storage. Small increase in sugar contents in neem leaf treated tomatoes as compared to control and other treatments (Figure 1) might be due to its slow ripening process and respiration [11]. Neem leaf extract integrated with 3% CO_2 storage slowed down the rate of respiration, transpiration and other metabolic changes due to its natural yield intrinsic activity or incorporation of antimicrobial compounds [12]. The maximum amount of sugars in untreated control might be due to rapid conversion of starch to sugars as a result of moisture loss and decrease in acidity by physiological changes during storage [13]. Our results are also in accordance with Melkamuet al [14]. Similar results were also quoted by Krammeset al [15], Opiyo and Ying [16] using different tomato cultivars.

3.2. Effects of Post-Harvest Bio-Chemicals on the Titratable Acidity of Tomatoes during Storage

In general, fruit titratable acidity tends to decrease and total soluble solid contents increase with maturation. Titratable acidity (TA) values of both the cultivars Sahil and Rio grande tended to reduce during storage under both storage conditions. Effect of interaction between bio-chemicals and CO_2 on the acidity of tomatoes was found highly significant. Acidity of neem leaf treated tomatoes with CO_2 storage decreases slowly as compared with that of control, Chitosan and *Aloe vera* treated tomatoes under same storage conditions (Figure 2). At the first day of storage, TA values of neem

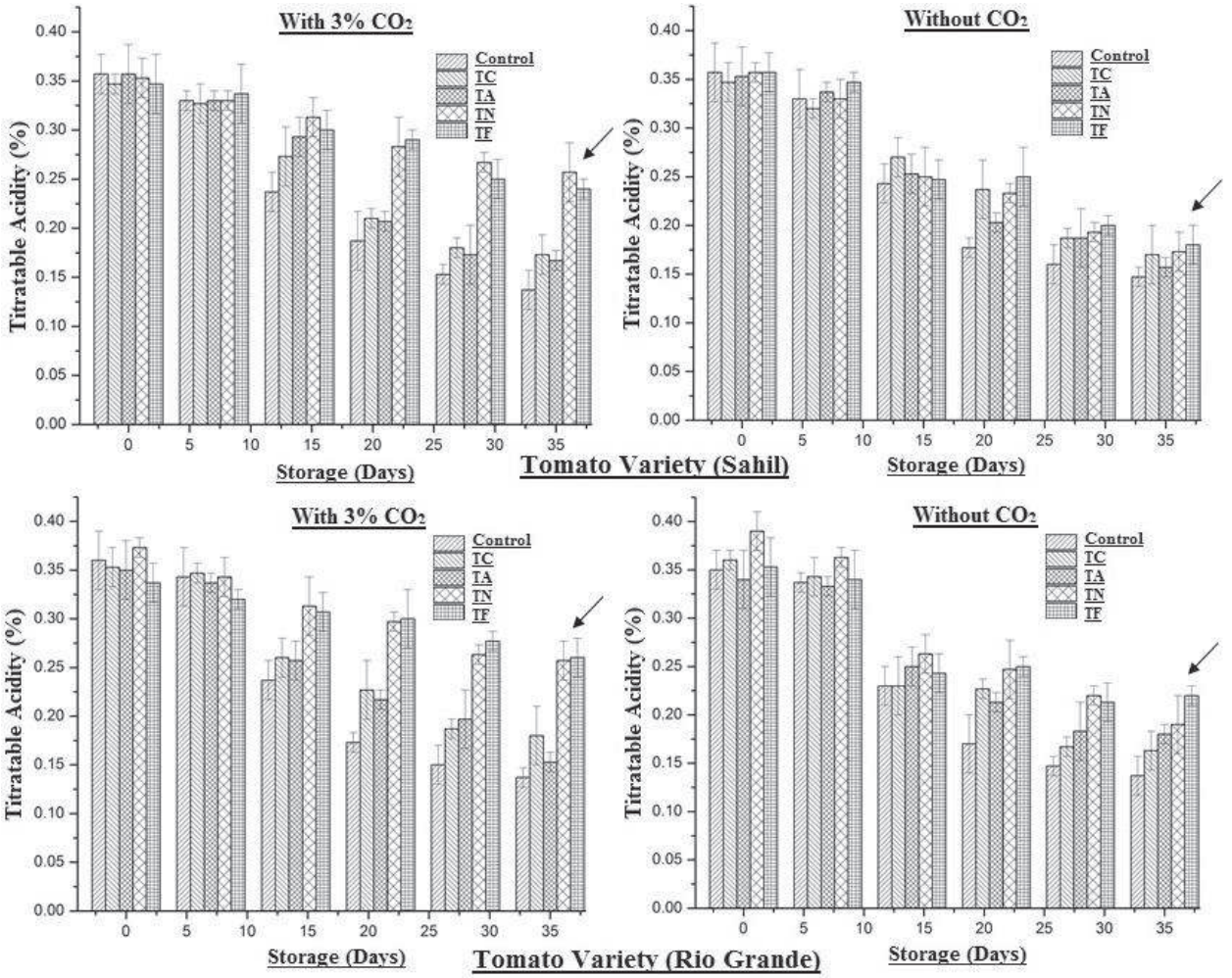

Fig. 2. Effects of post harvest treatments on the titratable acidity of the tomatoes during storage. Control: untreated tomatoes, TC: Tomatoes were treated with Chitosan, TA: Tomatoes were treated with Aloevera gel, TN: Tomatoes were treated with Neem leaf extract, TF: Tomatoes were treated with conventional fungicides thiophanate methyl.

leaf treated tomatoes with CO_2 were 0.35% that a decrease to 0.25% at 35 days while TA of control sample change from 0.35 to 0.13% during storage period of 35 days. The response of both cultivars Sahil and Rio grande in terms of TA change was found not significant. Slow reduction in titratable acidity of neem treated tomatoes stored with CO_2 may be due to the anti-microbial properties of neem leaf extract that slow down the microbial breakdown of organic acids. It is also supported by Castro et al. [17] who observed that the rate of reduction in titratable acidity in coated fruits compared to uncoated fruits is low due to restriction of oxygen availability that leads to reduced respiration rate. Our findings of this study regarding TA change were similar to the findings of Krammeset al [15]; Fernandez-Trujillo and Sanchez [18]; Opiyo and Ying [16] on tomatoes. It is also in agreement with Good enough and Thomas (1981) who found that titratable acidity was affected by CO_2 storage.

3.3. Effects of Post-Harvest Bio-Chemicals on the Color of Tomatoes During Storage

Surface color changes involve loss of chlorophyll, and synthesis of other pigments, such as carotenoids and lycopene, during the ripening period. Thus, color change is often used as an index of the degree of ripeness, and provides primary information about the physiological condition of the fruits [19]. The results of the present study prove that neem leaf extract apparently restrict the change in color from yellow to red and help to retard the senescence. Color a*/b* values increased with increasing USDA color

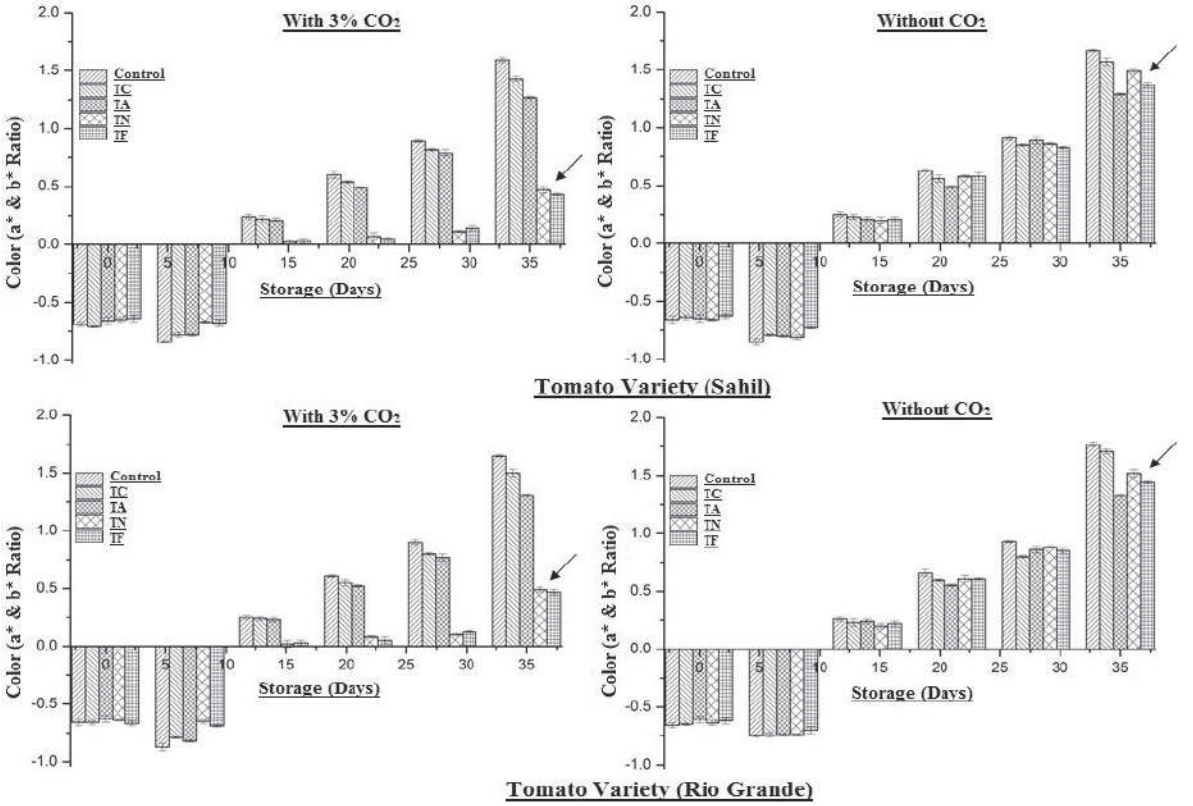

Fig. 3. Effects of post harvest treatments on the color of the tomatoes during storage. Control: untreated tomatoes, TC: Tomatoes were treated with Chitosan, TA: Tomatoes were treated with Aloevera gel, TN: Tomatoes were treated with Neem leaf extract, TF: Tomatoes were treated with conventional fungicides thiophanate methyl.

stages (Figure 3). Color a*/b* values of neem leaf treated tomatoes with 3% CO_2 were significantly differ from control, Chitosan and *Aloe vera* treated tomatoes except thiophanate treatment. Results of thiophanate treatment were also similar with neem leaf treatment at same storage condition. Effect of biochemical was found non-significant with respect to Variety. Effect of bio-chemicals on a*/b* values of Sahil and Rio Grande were almost similar. The color values at the breaker stage of tomatoes were also negative. Variation in color readings between maximum and minimum values increased during ripening of tomatoes. The color development rate of tomatoes increased with increasing maturation [20]. Neem leaf and thiophanate treated tomatoes stored at 3% CO_2 showed less color development as compared to all other treatments at same storage condition. Color a*/b* value of control of Sahil cultivar at the end of storage was 1.59 and 1.67 of Rio grande with 3% CO_2 while a*/b* values of neem leaf treated tomatoes were 0.47 and 0.49 of Sahil and Rio grande respectively. Results showed slow color development of neem leaf treated tomatoes under 3% CO_2 as compared to all other treatments. However thiophanate treated tomatoes showed the same results as compared to neem leaf with respect to color development. Slow color development in neem leaf and thiophanate may be due to the integrated action of the antimicrobial neem leaf extract and modified atmosphere that slow down ripening and respiration processes within the fruit. Color changes are well correlated with chlorophyll breakdown and carotenoid mainly beta-carotene accumulation in the plastids. Carotenes and xanthophylls, especially lycopene, oxidize during the storage and gradually change from bright red to dark brown. Our results are in accordance with the finding of Jiang and Li [21].

3.4. Effects of Post-Harvest Bio-Chemicals on the Respiration Rate of Tomatoes During Storage

Respiration, transpiration and ethylene production are the main factors contributing to the deterioration of fruits and vegetables [22]. Tomatoes treated with

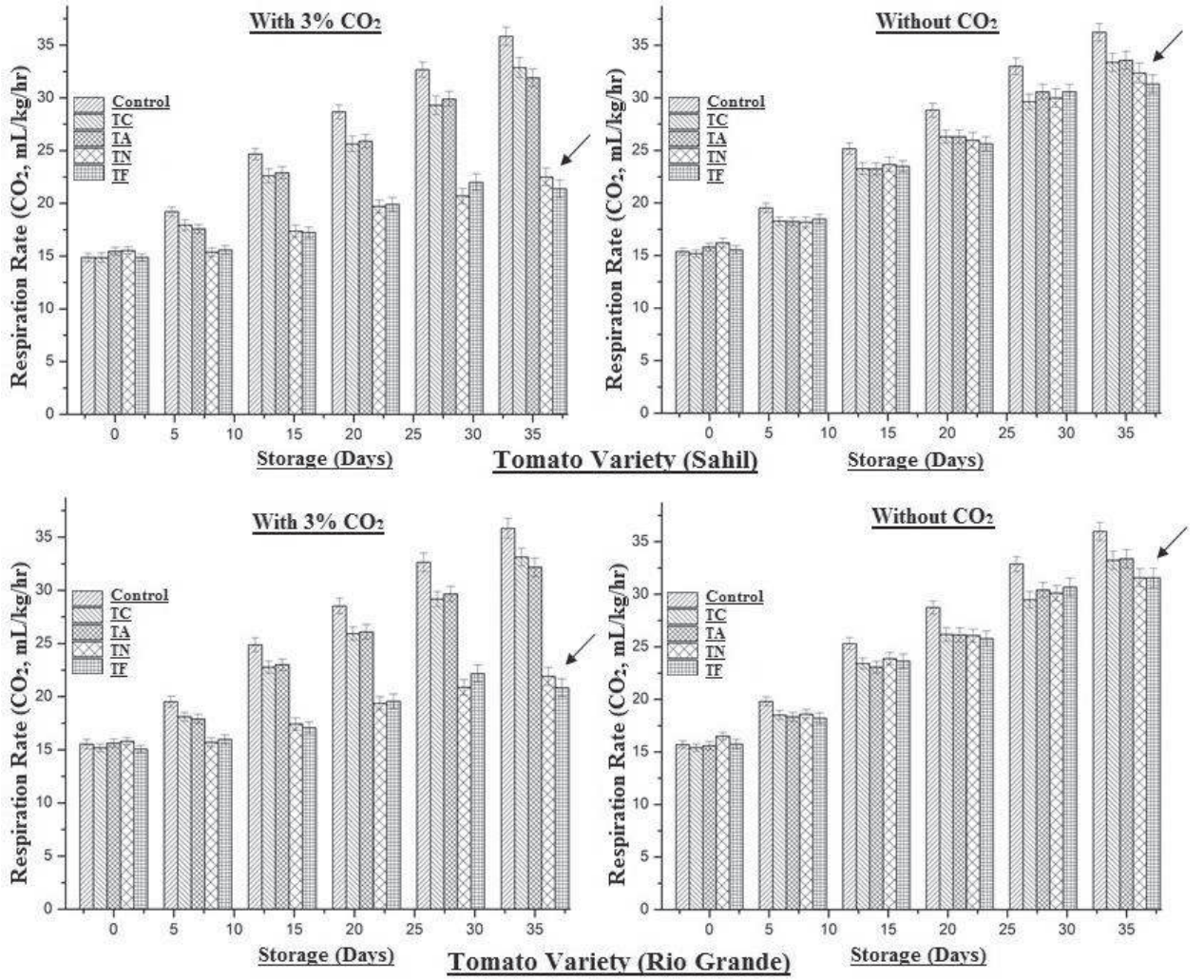

Fig. 4. Effects of post harvest treatments on the respiration rate of the tomatoes during storage. Control: untreated tomatoes, TC: Tomatoes were treated with Chitosan, TA: Tomatoes were treated with Aloevera gel, TN: Tomatoes were treated with Neem leaf extract, TF: Tomatoes were treated with conventional fungicides thiophanate methyl.

neem leaf extract and stored at 3% CO_2 significantly reduced respiration rates during storage as compared to control, Chitosan and *Aloe vera* treatments except thiophanate treated tomatoes at same storage condition (Figure 4). Neem leaf treated tomatoes stored with the CO_2 produced 15.53mL CO_2 $kg^{-1}h^{-1}$ at first day and 22.5mL CO_2 $kg^{-1}h^{-1}$at the last day of storage while control produced 14.87mL CO_2 $kg^{-1}h^{-1}$ at first day and 35.83mL CO_2 $kg^{-1}h^{-1}$at the last day of storage at same storage condition. Effect of biochemical on Sahil and Rio grande cultivars were found non-significant regarding respiration rate. Slow respiration of neem leaf treated tomatoes stored with CO_2 may be due to the anti-microbial properties that resist the senescence of tomatoes during storage. This may also be the result of the fact that reduction of O_2 supply to the fruit surface inhibits respiration rate [21]. The high organoleptic quality of fruit or vegetable products can be obtained by the control of ethylene production and respiration with controlled atmosphere. The respiration rate of fruits and vegetables is reduced by an atmosphere of high carbon dioxide and low oxygen which depress ethylene production, thus stop the ripening process [23]. The shelf life of fruits and vegetables is prolongedby reducing the rate of respiration with limited O_2that ultimately delay the oxidative breakdown of the complex substrates which make up the product. Use of modified or controlled atmospheres should be

considered as an interesting alternative to reduce respiration and ethylene production, maintain firmness and delay pathological decay. Knee [24] reported that respiration rate gives an indication ofthe rate of breakdown of respiratory substrates such asstarch, sugars and organic acids. This could have an implication towards better storage qualityof processed fruits as compared to the fresh market tomato varieties.

3.5. Effects of Post-Harvest Bio-Chemicals on the Ethylene Production of Tomatoes During Storage

Ethylene production of tomato fruit was also suppressed by application of neem leaf extract under 3% storage condition. The rate of ethylene release in the control tomatoes initially increased and reached a peak value of 9.87 µL kg-1 h-1 at 35 storage days. Initially the ethylene production was increasing rapidly, but at the end of storage ethylene production was slow down as shown (Figure 5). The ethylene production rate of neem leaf treated tomatoes with CO_2 storage was found significantly slower than control, Chitosan and *Aloe vera* treated tomatoes stored with and without CO_2. Neem leaf treated tomatoes produced 3 µL kg^{-1} h^{-1} at the first day while it reached to maximum 5.83 µL kg^{-1} h^{-1} at 35 days. Ethylene production of thiophanate was very close (5.9 µL kg^{-1} h^{-1})

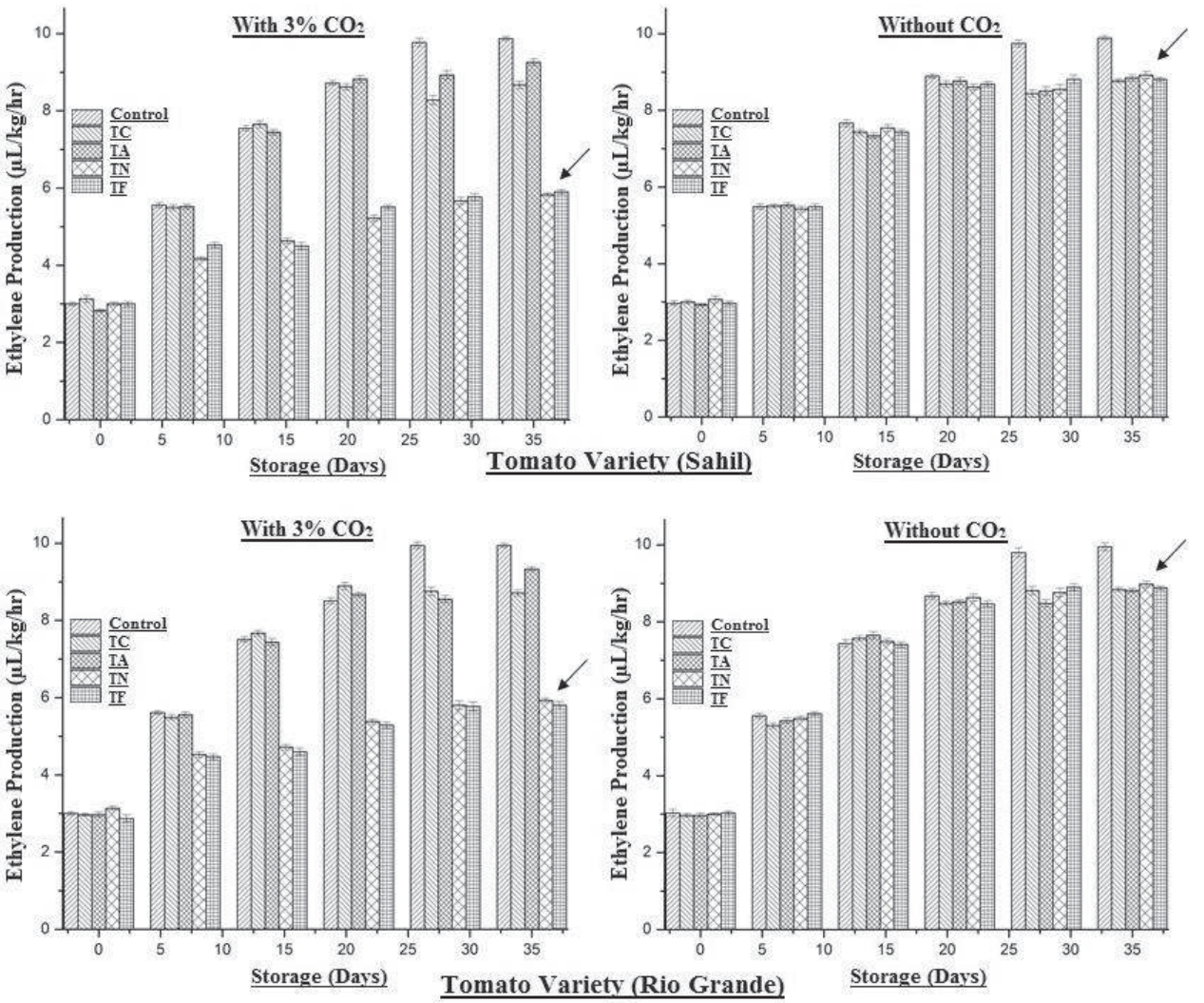

Fig. 5. Effects of post harvest treatments on the ethylene production of the tomatoes during storage. Control: untreated tomatoes, TC: Tomatoes were treated with Chitosan, TA: Tomatoes were treated with Aloevera gel, TN: Tomatoes were treated with Neem leaf extract, TF: Tomatoes were treated with conventional fungicides thiophanate methyl.

with neem leaf treated tomatoes (Figure 5). The results indicate that the interaction between neem leaf treatment and CO_2 storage condition has a significant effect on delay the tomato ripening as compared to all other treatments with and without CO_2. The effects of all bio-chemicals were found non-significant with respect to variety. Both Sahil and Rio grande showed the same results regarding ethylene production with and without CO_2. Limiting pericarp color reddening and ethylene production in tomato fruit lead to a delay in fruit ripening. Neem leaf application integrated with carbon dioxide could effectively retard pericarp color reddening and suppress ethylene production in tomato fruit during storage, which indicates that neem leaf treatment with CO_2 is beneficial in delaying fruit ripening, resulting in higher resistance to the fungal decay as compared to the controls, *Aloe vera* and Chitosan. Low O_2 or elevated CO_2 concentrations may reduce ethylene synthesis directly by affecting the activity of the softening enzymes, particularly 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase by inhibiting ethylene binding to a receptor that triggers autocatalysis [25]. Results are in accordance with the finding of Zapata et al[26], Mejia-Torres et al[27] and Lai et al [28].

4. CONCLUSIONS

Demand for chemical free food products have been well raised and bio-pesticides gradually replacing the highly toxic pesticides in the market. In the present study it was concluded that the neem leaf extract integrated with 3% CO_2 atmosphere can decrease the physiological process during storage and thus reduces the post-harvest losses effectively in tomatoes. Moreover, neem leaf extract coating can be used instead of conventional post-harvest fungicide during storage of tomatoes to control the post-harvest losses.

5. ACKNOWLEDGEMENTS

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Research Article

Chromium Induced Elevated Glutathione S-transferase Activity in *Cirrhina mrigala*

Samreen Gull[1], Sajid Abdullah[1], Huma Naz[2*], and Khalid Abbas[1]

[1]Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad
[2]Department of Zoology, Cholistan University of Veterinary and Animal Sciences Bahawalpur

Abstract: In aquatic bodies, heavy metals contamination is a most important issue all over the world. Heavy metals are persistence in nature and can store in aquatic environment. These metals have ability to induce oxidative stress and altered the enzymes activities. Therefore, in current investigation the activity of glutathione S-tranferase (GST) in hepatic, renal and cardiac tissues of fish, *Cirrhina mrigala* under various sub-lethal concentrations (1/3rd, 1/4th and 1/5th of LC50) of chromium (Cr) was evaluated. The fish was exposed to chromium for 21-day and sampling was done on weekly basis. Results demonstrated that the GST activity was significantly augmented in hepatic, renal and cardiac tissues of exposed fish in relation to control. Comparison among organs revealed that the maximum GST activity was observed in hepatic tissues of fish followed by renal and cardiac tissues. The GST activity gradually augmented with increasing the concentration and duration of exposure. In conclusion, the use of GST enzyme as biomarkers is not a new approach but can be used successfully to assess the toxicity associated with metals in aquatic animals.

Keywords: Fish, Chronic exposure, Antioxidant enzyme, Organs.

1. INTRODUCTION

Pollution of aquatic ecosystems has been increased with the rapid expansion of industries and anthropogenic activities [1]. Among these pollutants, heavy metals are unique because they cause adverse effects on aquatic plants and animals even at lower concentration [2]. Metals are non-biodegradable, have ability to a amass in aquatic animals, especially fish, [3] become harmful and at the end pass to the other living organism like humans who eat these aquatic animals as food [4]. Toxicity of metals in aquatic environment influenced by many factors includes solubility, type, and complexation. The metals interaction can alter their toxicity to aquatic life either negatively and positively [5].

Chromium in the aquatic bodies enter through waste released from textiles, leather tanneries, metal finishing, photographic and pharmaceutical industries, electroplating, mining, ceramic, dyeing and printing industries etc. [6-7]. The lethal effects of chromium are contact dermatitis, organ system-toxicity, allergy and alterations in the histology of various parts [8].

Heavy metals have ability to produce oxidative stress by stimulating the formation of reactive oxygen species (ROS), like superoxide radical, hydroxyl radical and hydrogen peroxide, via Haber–Weiss and Fenton reactions resulted in oxidation of macromolecules such as lipids, proteins and nucleic acids, often leading to damage the structure of cell [9-10]. Aquatic animals have antioxidant defense scheme to overcome the injurious effect of ROS. This system comprises enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione S-transferase (GST) and glutathione reductase (GR) [11-12]. Glutathione S-transferase (GST) belongs to the family of detoxifying enzymes, plays a significant role in metabolism of xenobiotics, catalyzing reactions of binding xenobiotics with GSH [13]. GST is

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* Corresponding Author: Huma Naz; dr.humanaz98@gmail.com

a Phase II system enzyme which decreases the harmful effects of endo- and exogenous substances. Other than this GST performed many function in metabolism includes degradation of tyrosine, biosynthesis of hormone, dehydro-ascorbate reduction and peroxide breakdown [14].

Biochemical parameters are an important approach to assess the harmful effect in exposed fish. Oxidative stress biomarkers are now rapidly used for environmental monitoring program in the field of ecotoxicology [15].Therefore, this experiment was performed to elucidate the sub-lethal effects of chromium on glutathione S-transferase activity in *Cirrhina mrigala.*

2. MATERIALS AND METHODS

2.1 Experimental Trail

This experiment was performed at Fisheries Research Farm department of Zoology, Wildlife and Fisheries, Universities of Agriculture, Faisalabad. Freshwater fish, *C. mrigala* were selected for this experiment. The juveniles of *C. mrigala* were obtained from Fish Seed Hatchery Faisalabad and immediately shifted to Fisheries Research at University of Agriculture, Faisalabad. Prior to experiment, *C. mrigala* fingerlings were kept in cemented tank for two weeks to acclimatize the laboratory condition. As the acclimatization period completed, fish were transferred to 75-litre glass aquarium. Each aquarium contained ten fishes. Metal solution was prepared by using chemically pure chloride compounds of chromium. The 96-LC_{50} for *C. mrigala* was calculated as 68.22 mgL^{-1} reported by Batool and Javed [16]. The lethal value was divided by 3, 4 and 5, and got sub-lethal concentration for *C. mrigala* about 22.74 ($1/3^{rd}$), 17.06 ($1/4^{th}$) and 13.64 ($1/5^{th}$) mgL^{-1}. Fish, were exposed to sub-lethal concentrations for 21 days and sampling was done on weekly basis.

2.2 Physico-Chemical Parameters of Water

During the whole experiment, total hardness, pH and temperature of water were kept stable as 225 mg L^{-1}, 7.25 and 30°C. However, other variables (Ca, Na, Mg, CO2, EC, NH3 and K) were also calculated and maintained on daily basis [17].

2.3 Estimation of GST Activity

2.3.1. Organ Homogenate

The enzyme GST was isolated from the hepatic, renal and cardiac tissues of *C. mrigala.* The organs were weighted and add cold 50mM Tris HCL buffer (pH 7.4) containing 0.2 M sucrose, 4 times greater than the weight of organ (1:4 w/v). The organs were homogenized for 15 minutes using a pestle and mortar. After that, organ homogenates were centrifuged for 15 minutes at 10,000 rpm and 4°C. After centrifugation process, clear supernatants were stored at-80°C for enzyme essay while residue was discarded.

2.3.2. Enzyme assay

The reaction mixture of 3ml was contained 2.4 ml of 0.3 M potassium phosphate buffer (pH 6.9), 0.1ml of 30m M CDNB, 0.1ml of 30mM GSH and 0.4ml of enzyme supernatant. Absorbance was read at 340nm against the reagent blank on spectrophotometer minute [18].

2.4. STATISTICAL ANALYSIS

Data obtained from this study were analyzed by applying analysis of variance to get statistical differences among variables. The value of $p > 0.05$ were consider as statistically non-significant. MS excel was used to draw graphs.

3. RESULTS AND DISCUSSION

The level of GST in hepatic, renal and cardiac tissues of chromium exposed fish was significantly augmented as compared to control (Figure1-3). Tissue specific response showed that maximum GST level was observed in hepatic tissue (Figure 4). Maximum GST activity was observed due to $1/3^{rd}$ of LC_{50} concentration of chromium followed by the order: $<1/4^{th} <1/5^{th} <$control. The GST activity was gradually augmented as the duration of exposure increased. The GST activity was maximum after 21-day of exposure to metal followed by 14- and 7-day. Farombi et al [19] also determined the increased GST activity in kidney, heart and liver of barbell catfish exposed to heavy metals (Cd and Cu). Yuan et al. [20] noted the Cr induced GST in whole body of *Gobiocypris raru*. Abdullah et al [21] reported the higher level of GST in liver and kidney of *Channa striata* due to lead+nikel

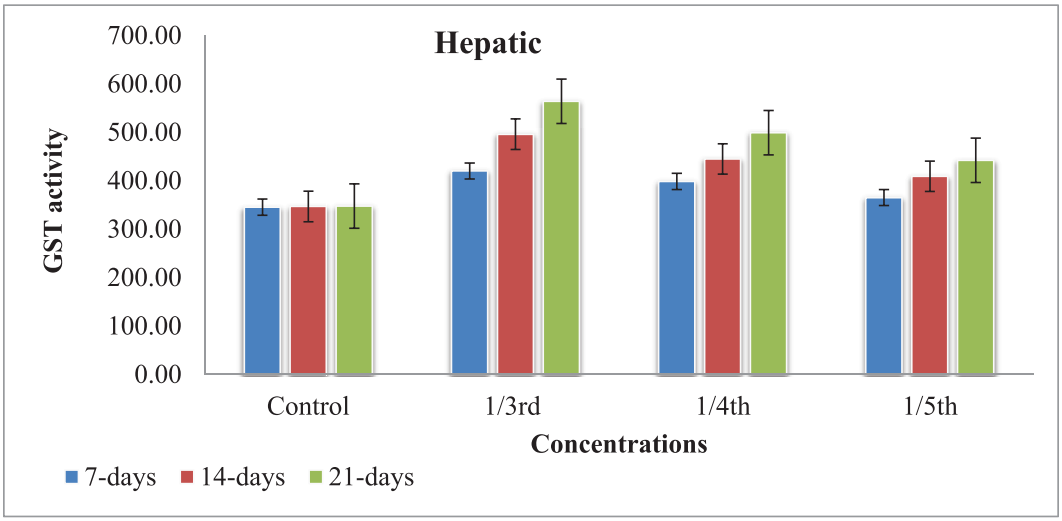

Fig. 1. GST activity (U/ml) in hepatic tissue of *C. mrigala* exposed to sub-lethal concentrations of chromium

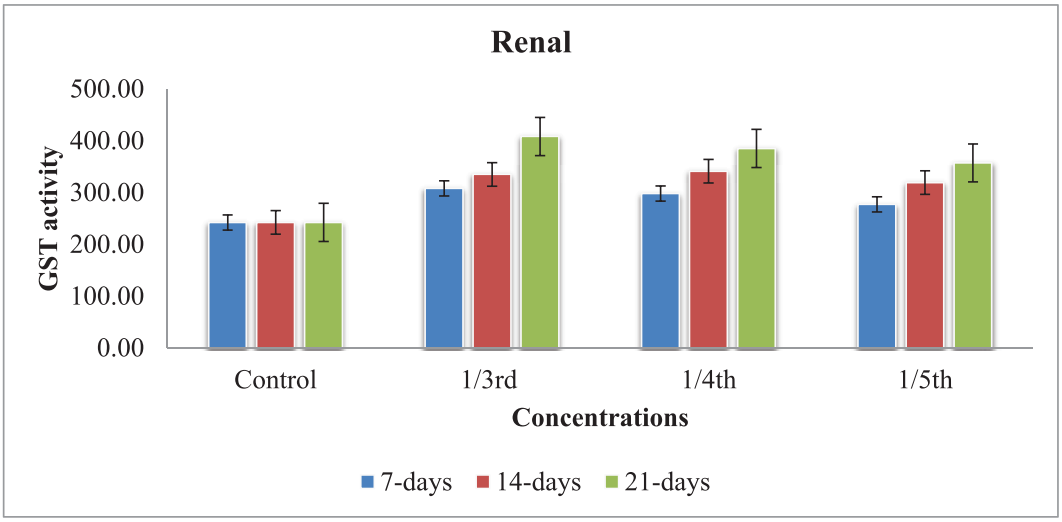

Fig. 2. GST activity (U/ml) in renal tissue of *C. mrigala* exposed to sub-lethal concentration of chromium

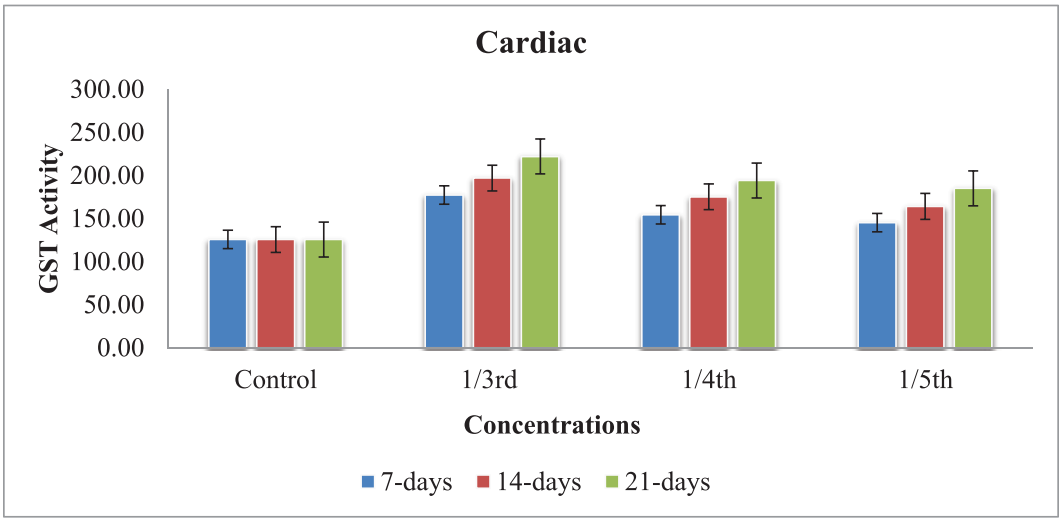

Fig. 3. GST activity (U/ml) in cardiac tissue of *C. mrigala* exposed to sub-lethal concentration of chromium

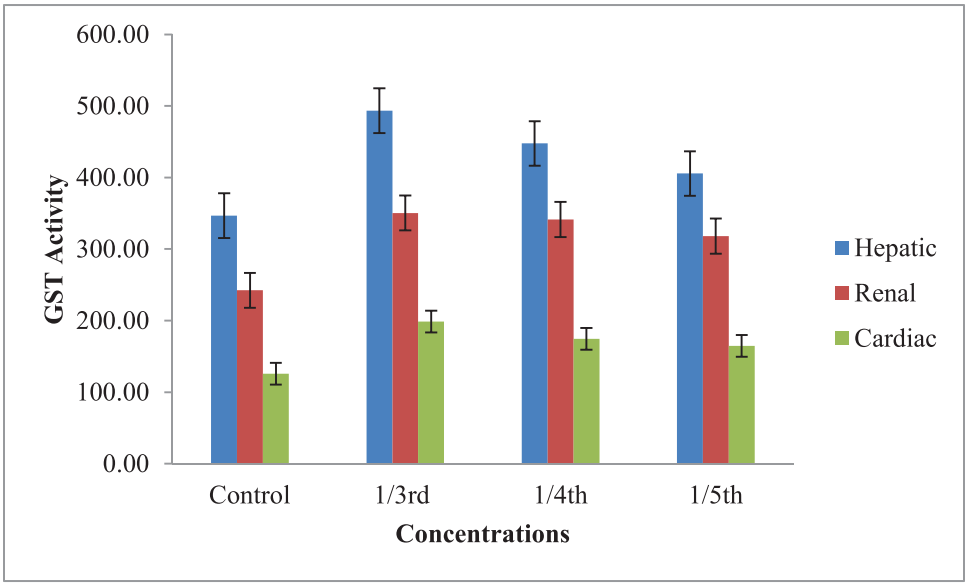

Fig. 4. Comparison among organs for GST activity in *C. mrigala* exposed to different sub-lethal concentrations

exposure.

Vinodhini and Narayanan [2] also support this study who observed the increased GST activity in common carp exposed to various concentrations of heavy metals (Cd, Ni, Pb and Cr) for 32-day. These results suggest that increased activity of enzyme may prevent the fish from damage of free radical mechanism. GST is a Phase II detoxifying enzyme, transferred the xenobiotic to harmless substances. However, antioxidant enzymes eliminate the ROS from the cell [22-23]. Previous report mentioned that GST significantly increased in the presence of heavy metals [24] mainly depends on the type of tissue and species.

According to Saliu and Bawa-Allah [25] exposure of zinc chloride (ZnCl2) significantly accelerated the hepatic GST activity of *Clarias gariepinus*. Batool et al. [26] noted the significant induction of GST in liver of *Wallago attu* in a duration and concentration dependent manner. Eleyele et al [27] noted the elevated GST level in liver of *Clarias gariepinus* exposed to effluent contained heavy metals discharged from a pharmaceutical industry. According to Arafa et al [28] heavy metals (lead, cadmium, copper, zinc, iron, nickel and chromium) contamination in Ismailia channel water significantly induced the GST activity in liver of the fish *Clarias gariepinu.* Exposure of Cadmium elevated the GST activity in hepatic and renal tissues of *Arius arius* [29]. According to Aladesanmi et al [30] higher concentration of Pb and Cr accelerated the GST activity in the fish, *Clarias gariepinus* tissue (liver, gills, fins and muscle). GST was significantly higher in liver and kidney of *Channa punctatus* inhabiting heavy metals (Mn, Fe, Zn, Co, Ni, Cu and Cr) loaded waste water [31].

4. CONCLUSION

The current study concluded that the sub-lethal exposure of chromium at various concentrations can alter the activity of glutathione S-transferase enzyme. Among tissues, hepatic tissue showed maximum activity due to its role in detoxification of toxicants. Results of this work also suggest that GST can be used as an important biomarker for evaluating the metal toxicity in aquatic ecosystems. It was also concluded that the chromium concentration above threshold level could be lethal not only to aquatic life as well as human health. Therefore, preventive measures should be taken to minimize the concentration of metals contamination in aquatic bodies.

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Research Article

Contemplating Toxicity of Atrazine on Lipid Profile of Fresh Water Fish *(Ctenopharyngodono idella)*: An Experimental Approach

Ahsan Khan[1*], Nazish Shah[1], Mukhtar Alam[2], and Khaliq Ur Rahman[3]

[1]Department of Zoology, University of Swabi, KP
[2]Department of Agriculture, University of Swabi, KP
[3]Department of Chemistry, University of Swabi, KP

Abstract: Productivity of agriculture is dependent upon the utilization of herbicides but they find avenues to get into water bodies, affecting aquatic fauna, particularly fish. The current effort accordingly focused to contemplate toxicity of sub lethal concentration of herbicide i.e. atrazine by estimating the lipid profile that included Cholesterol (CH), Triglyceride (TG), High density lipid (HDL) and Low density lipid (LDL) of freshwater grass carp, (*Ctenopharyngodon idella*) for (24, 48, 72, 96, 240, 360 and 600 hrs) under the dose (15, 13, 10, 08, 06, 04 and 02 µl L-1) respectively. Merck micro lab 300 biochemistry analyzer was used for analyzing lipid profile. Acute toxicity exposure explored that all components of total lipids documented decline in concentration against each time period and maximum decrease was observed against 72h exposure respectively. Similarly chronic toxicity exposure also indicated the decline against each time period and maximum decline in CH, TG and LDL was observed against 600h while HDL showed reduction in concentration against 360h exposure respectively. In all component of the lipid profile, significant decline in concentration was observed as; $P<0.05$, $P \leq 0.01$ and $P \leq 0.001$, specifically in acute toxicity groups as compared to chronic toxicity groups thus showing undesirable effects of on aquatic fauna present inside water bodies.

Keywords: Acute toxicity, Grass Carp, Cholesterol, Triglyceride.

1. INTRODUCTION

The water pollution has become the growing concern in the recent years mainly the toxic pollutants that have altered the ecological balance by accumulating in the aquatic environment, decreasing the productivity and fecundity of aquatic life thus, effecting the humans that rely on those organisms as a major source of protein [1]. All animals are affected by such toxicant but fish the most vulnerable among these organisms is heavily exposed to different toxicants with no escape from the pollution and serves as the most important bio monitors for estimation of metal pollution level [2].

Herbicide plays an important role in agriculture production and considered as an important part of agronomy [3]. The agriculture products can be increased by implanting the herbicide which ends in killing the undesired plants and leaving the desired crop [4]. But the implementation of this herbicide also disturbed the ecosystem, i.e. adherence of herbicide particles to wind ends in wind pollution thus effecting wildlife and birds. Adherence to herbs thus affecting the domesticated animals viz grazing as food chain and adherence of particles to soil can affects the soil microorganism. Furthermore, these particles viz wind, soil erosion and surface runoff these particles finds the ways to aquatic bodies thus effecting the aquatic organisms particularly fish biodiversity and has been threatened by producing variation in its physiological system and similarly accumulating of these toxic substances in tissues finds it end in human bodies through food chain. [5].

Herbicide alters the ecosystem of every fauna present inside aquatic bodies, particularly fish [6]. Alteration in the ecosystem effects the enzymatic and hormonal activities of fish thus influences its physiology, behavior, growth as well as

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*Corresponding Author: Ahsan Khan; ahsan@uoswabi.edu.pk

reproduction [7]. The high toxic media also ends in excess amount of the mucus which in combination with water produces unpleasant smell, rapid movement of the body, degenerating of fin rays that led to losing of the balance, suffocation that effect the respiration, changes of body color etc [8].

Atrazine was formulated in 1958 as the second series of 1, 3, 5-triazines. It was the succeeding mostly used herbicide after glyphosate in U.S during 2014 and used to avoid the pre-and post-emergence broadleaf weeds or grassy weeds in different crops for example, maize (corn), sorghum, sugarcane, pine, lupins, eucalyptus plantations and triazine tolerant (TT) canola [9]. Atrazine the most important endocrine disruptors are banned in the US and other countries while some countries used it to reduced the pre and post growth of the weed in different crops [10]. Atrazine after being applied to soils it does not break down within a few weeks because of its half-life that ranges from 13 to 261 days in soil. Because of high mobility i.e. about 600 miles from the point of application it has been spotted inside aquatic bodies [11]. Different analysis have proved the harmful effects of atrazine on fish hematological parameters, locomotor activities, metabolism, immune responses, osmoregulatory disturbance, oxidative stress and reproduction of fishes [12-18]. In this regard the present study was design to scrutinize the toxicity of atrazine to grass carp *(Ctenopharyngodon idella)* by subsequently finding out its acute (24, 48, 72 and 96hrs) and chronic toxicity (240, 350 and 600 hrs) via undertaking the evaluation of lipid profile including cholesterol, triglyceride, high density lipid and low density lipid.

2. MATERIAL AND METHODS

2.1 Maintenance of Experimental Fish

Grass carp (*Ctenopharyngodon idella*: 8.5 ± 5.5 cm; 9.5 ± 6.5g) were procured from carp hatchery of Mardan and Peshawar and acclimatized for two weeks in aquarium having tap water and then were shifted to experimental tanks. Both in acclimatization and experimentation tanks fish were fed with commercial carp pellet diet (Oryza Organics, Pakistan) on each alternate day. Physiochemical parameters of water were also recorded on every alternate day during acclimation and exposure period and were found in permissible limits as per the recommended values of APHA and American Public Health. Different water quality parameter was also checked on every alternate day including pH (normal), temperature (normal), total hardness; 95mg/l, calcium hardness; 61.6mg/l, magnesium hardness; 35mg/l, water conductivity; 431µS/cm, DO; 7.37ppm, TS; 321mg/l, TDS; 221mg/l, TSS; 100mg/l, total alkalinity; 163.3mg/l and chloride concentration; 20.3mg/l and all these values indicated normal parameter concentrations.

2.2 Experimental Design

Fish were divided in to four groups of 10 fish per group for acute toxicity analysis and exposed to herbicide (atrazine) for 24, 48, 72 and 96 hours respectively. Group1 to 4 were treated against dose of $15\mu lL^{-1}$ for 24h, $13\mu lL^{-1}$ for 48h, $10\mu lL^{-1}$ for 72h and $08\mu lL^{-1}$ for 96h respectively. Similarly for chronic toxicity fish were divided in to three groups of 10 fish per group and each group was exposed against dose of 6 μlL^{-1} for 240h, 4 μlL^{-1} for 360 and 02 μlL^{-1} for 600h respectively.

The experiment was conducted in semi-static conditions, following OECD guideline number 203 [19]. After the stipulated time, three fish were randomly selected and anesthetized using clove oil [20]. The anesthetic was prepared fresh by dissolving clove oil into absolute alcohol (Merck, Germany) in a ratio of 1:2.

2.3 Blood Collection and Preservation

Samples of blood were collected from caudal vein of fish and sometimes from direct puncturing of fish heart. The blood was obtained with the help of heparinized hypodermic syringes that contains heparin for avoiding blood clot [21-22]. After collection of blood, the tubes were kept in ice box and then were shifted to lab for further analysis. EDTA tubes and gel tube were utilized for storage of blood samples and serum was obtained from blood viz centrifugation at 3000rpm.

2.4 Estimation of Lipid Profile and Statistical Analysis

Merck micro lab 300 biochemistry analyzer was used for analyzing lipid profile. Results were statistically reported by SPSS software.

3. RESULTS

Cholesterol concentration during acute and chronic toxicity evaluationagainst atrazine declined. Maximum highly declined in concentration (P≤0.001) was noted after exposing fish for 48 and 72h. However, a slight rise in concentration was noted with exposure for 96h, but the decline in concentration was highly significant (P≤0.01) as shown in the table 2 and fig.1. Likewise, during chronic toxicity highly significant decline (P≤0.01) in concentration was noted after exposure for 360h while maximum highly significant decline (P≤0. 001) in concentration was observed after 600h exposure as evidenced in the table 3 and fig. 1.TG concentration was a highly significant decline (P≤0.01) after exposure of fish for 72hrs during acute toxicity as noted in table 2 and fig.2 while during chronic toxicity TG concentration was highly significant decline (P≤0.01) after exposure for 600h as shown in the table3 and fig. 2.

Highly significant decline (P≤0.01) was observed after exposure for 48hrs while maximum highly declined (P≤0.001) was observed in HDL after exposure for 72hrs and 96hrs during acute toxicity, as indicated in the table 2 and fig. 3. During chronic toxicity HDL concentration was a highly significant decline (P≤0.01) after exposure for 360 and 600h respectively as indicated in the table3 and fig. 3. Similarly LDL concentration was highly significant declined (P≤0.01) after exposure of fish for 24 and 48h respectively, while maximum highly significant decline (P≤0.001) was noticed

Table 1. Lipid Profile concentration of control group

S.No.	Lipid Profile	Fish 1	Fish 2	Fish 3	Mean ± SD	Standard error of mean
1	Cholesterol (mmol/L)	180	186	184	183.3± 3.0	1.7
2	Triglyceride (mmol/L)	65	60	63	62.3 ±2.5	1.4
3	HDL (mmol/L)	148	140	144	144 ±4.0	2.3
4	LDL (mmol/L)	35	40	44	39.6 ±2.6	1.5

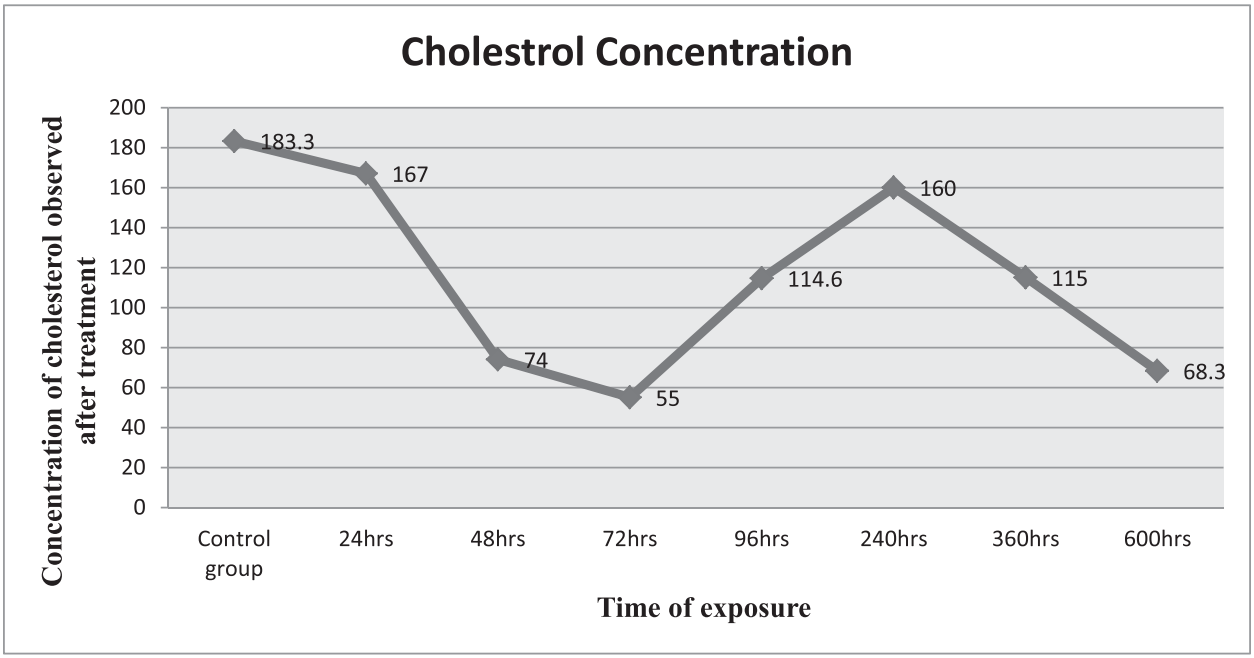

Fig. 1. Comparison of cholesterol concentration between control group and treated groups (acute and chronic toxicity)

Table 2. Lipid Profile concentration of control group

S.No.	Lipid Profile	Hours of treatment	Fish 1	Fish 2	Fish 3	Mean ± SD	Standard error of mean	Paired T test value	Significant Value (P)
1	**Cholesterol** (mmol/L)	24hrs	178	160	165	167.6±9.2	5.3	2.19	0.15NS
		48hrs	77	78	67	74±6.0	3.5	26.69	0.001***
		72hrs	52	58	55	55±3.0	1.73	385.00	0.000***
		96hrs	114	110	120	114.6±5.0	2.90	18.49	0.003**
2	**Triglyceride** (mmol/L)	24hrs	60	54	50	54.6±5.0	2.90	3.17	0.086NS
		48hrs	45	50	50	48.3±2.88	1.66	4.83	0.04 NS
		72hrs	26	28	32	28.6±3.6	1.76	13.52	0.005**
		96hrs	60	62	52	58±5.2	3.0	1.24	0.34 NS
3	**HDL** (mmol/L)	24hrs	131	140	136	135.6±4.5	2.60	1.69	0.23 NS
		48hrs	80	90	92	87.3±6.4	3.71	9.94	0.01**
		72hrs	30	35	36	33.6±6.80	3.92	28.07	0.001***
		96hrs	53	40	56	49.6±8.5	4.91	27.10	0.001***
4	**LDL** (mmol/L)	24hrs	35	34	36	35±4.16	2.40	1.94	0.019**
		48hrs	18	18	24	20±3.4	2.0	13.53	0.005**
		72hrs	15	20	22	19±3.6	2.08	31.00	0.001***
		96hrs	35	40	40	38±2.3	1.33	1.0	0.423 NS

[Significant Value =P<0.05] [High Significant value=P≤0.01] {Maximum highly significant value= P≤0.001] [Non Significant Value= P>0.05]
[Significant=*] [Highly Significant =] [Maximum highly significant value=***] [Non Significant= NS**

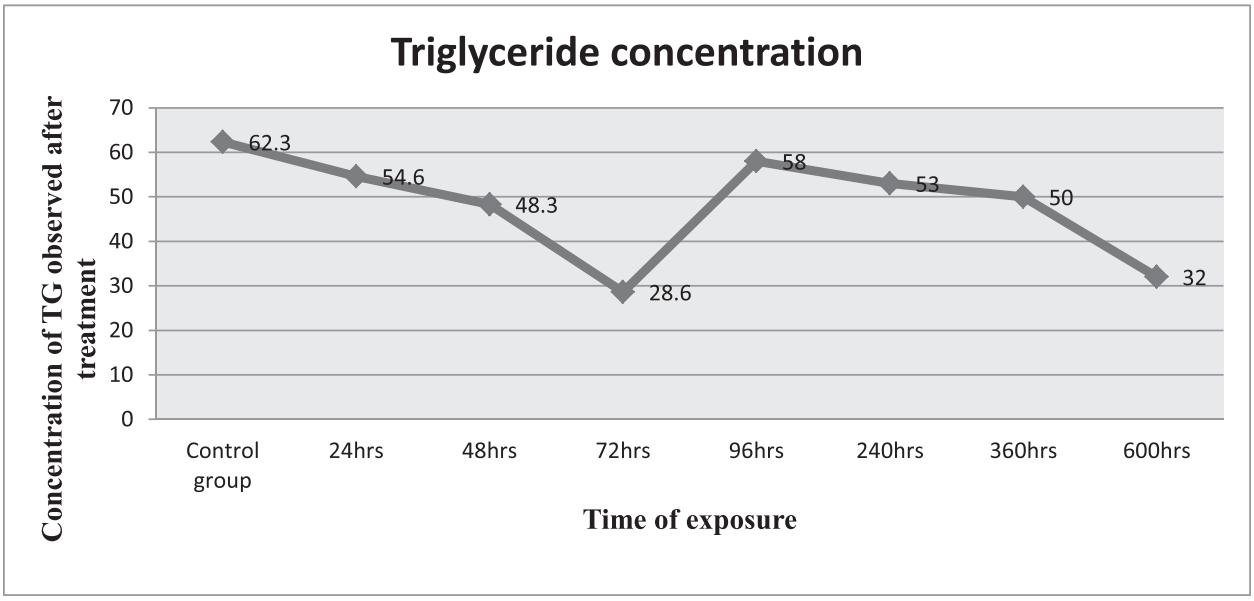

Fig. 2. Comparison of TG concentration between control group and treated groups (acute and chronic toxicity)

Table 3. Lipid Profile concentration of control group

S.No.	Lipid Profile	Hours of treatment	Fish 1	Fish 2	Fish 3	Mean ± SD	Standard error of mean	Paired T test value	Significant Value (P)
1	**Cholesterol** (mmol/L)	240hrs	160	155	165	160±6.65	3.84	6.07	0.26NS
		360hrs	120	115	110	115±7.37	4.25	16.05	0.004**
		600hrs	60	70	75	68.3±5.56	3.21	35.77	0.001***
2	**Triglyceride** (mmol/L)	240hrs	55	54	50	53±2.6	1.52	4.76	0.41 NS
		360hrs	50	52	48	50.0±4.04	2.33	5.42	0.032NS
		600hrs	30	28	38	32±5.2	3.05	10.31	0.009**
3	**HDL** (mmol/L)	240hrs	108	110	100	106±5.2	3.0	9.12	0.012*
		360hrs	80	92	83	85±6.2	3.60	10.06	0.01**
		600hrs	75	74	80	76.3±3.2	1.85	24.80	0.002**
4	**LDL** (mmol/L)	240hrs	36	34	38	36±2.0	1.15	1.57	0.25NS
		360hrs	28	18	22	22.6±5.0	2.90	3.40	0.07NS
		600hrs	20	20	22	20.6±1.15	0.66	9.12	0.01**

[Significant Value =P<0.05] [High Significant value=P≤0.01] {Maximum highly significant value= P≤0.001] [Non Significant Value= P>0.05]
[Significant=*] [Highly Significant =] [Maximum highly significant value=***] [Non Significant= NS]**

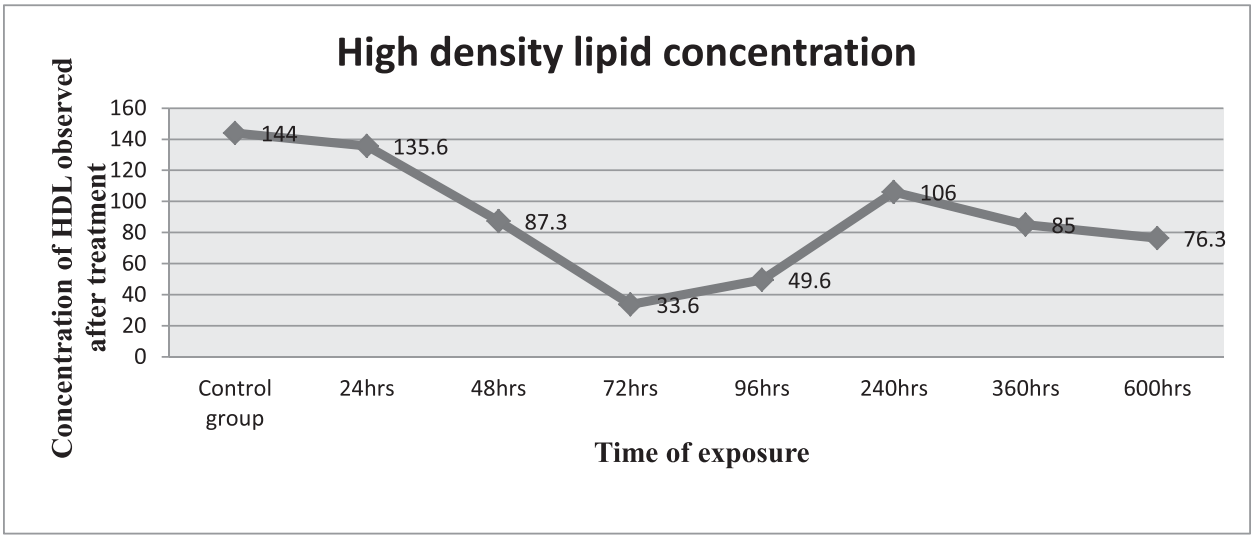

Fig. 3. Comparison of HDL concentration between control group and treated groups (acute and chronic toxicity)

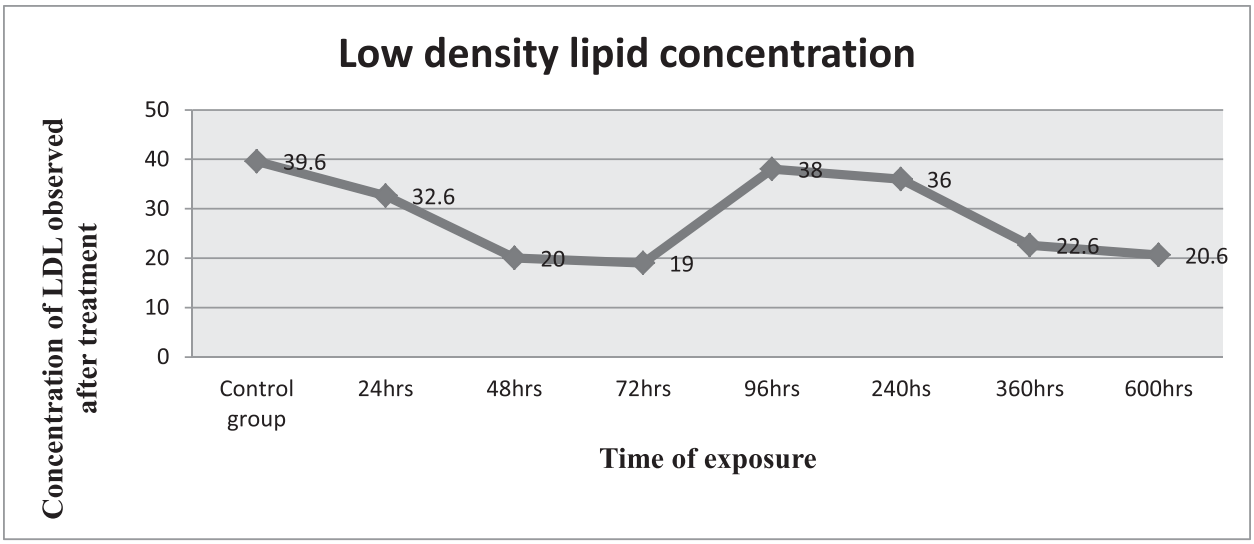

Fig. 4. Comparison of LDL concentration between control group and treated groups (acute and chronic toxicity)

after exposure for 72hrs during acute toxicity, as shown in the table 2 and fig. 4. During chronic toxicity highly significant decline ($P \leq 0.01$) was observed after exposure for 600h as shown in the table 3 and fig. 4.

Present findings indicated that lipid profile of grass carp was declined against various doses of atrazine in both acute and chronic toxicity and difference was observed in significant ($P < 0.05$), highly significant ($P \leq 0.01$), and maximum highly significant ($P \leq 0.001$) when herbicide treated group was compared with the control group and comparatively effect was more pronounced in the acute toxicity group as compared to chronic toxicity groups.

4. DISCUSSION

The most common cause of water pollution is runoff of domestic and industrial waste that is directly released into streams or ponds without treatment. Waste is consisted of various pollutants including; heavy metals, radioactive substances, herbicides and corrosive substances like acids and bases [23]. However, in Pakistan, another prominent source of aquatic pollution is agriculture industry; where growers use herbicides to manage herbs (unwanted plants) which obstruct the growth of undesired plants. But on the rear end, these herbicides find avenues to water bodies thereby affecting aquatic fauna. In response to a stressor such as herbicide exposure, the fish undergo a series of biochemical and physiological alteration in an effort to cancel the challenge imposed on them. Therefore, blood parameters such as hematological and biochemical indices can serve as important markers for diagnosing the structural and functional status of fish exposed to herbicide [24].

Atrazine affects the fish in different ways like alteration in blood parameters of *Cyprinus carpio*, fat oxidation and antioxidant enzyme of *Channa punctatus*, after exposing to different concentration of atrazine herbicide [25]. Atrazine is toxic to aquatic animals and exposure of fish to atrazine result in biochemical parameters alteration, behaviorally abnormality, structurally deformation, causing stress, on reproduction, on the immune system by quantifying white blood cells etc [26-29].

Lipids serve as vital source of energy by providing structural components for reproductive growth [30]. In the present study, grass carp *(Ctenopharyngodon idella)* was exposed to various doses of atrazine for short term (acute toxicity) as well for the long term (chronic toxicity) to scrutinize lipid profile concentration. The results indicated markedly decline (denoted by $P < 0.05$, $P \leq 0.01$ and $P \leq 0.001$) in a concentration of lipid profile, including cholesterol, triglyceride, high density lipids and low density lipids. For justification of the present study, reduced level of lipid content in the organs of the fish after 96 hours exposure to atrazine were found to be 1.56±0.13mg/100mg wet tissue, 1.90±0.04 mg/100mg wet tissue and 1.23±0.19 mg/100mg wet tissue in the gills, liver

and kidney respectively. It might be ascribable to the diminution in the absorption of carbohydrate and protein, resulting in the depletion of energy during toxic stress, which contributes to the degradation of lipid to combat the required energy.

As the level of the protein and carbohydrate absorption decreases the lipid level also decreases due to lipid metabolism to match the needed energy during the stress condition [31]. The present study was in agreement with El-Sayed et al [32] who illustrated that the decrease in body lipid in appropriate habitat was a direct of utilization of body fat as an energy supply to meet the increase in physiology demands. So, to manage with stress situation the fish utilized the fats in the body to raise energy to overcome such stress situation.

Khan et al [33] observed effects of cadmium on biochemical contents in liver and ovary of *Garramalaya* and found that a substantial decrease in cholesterol and stated that this may be due to general damage. Shakooriet al [34] examined the result of sub lethal doses of fenvalerate on the blood, liver and muscles of fish *Ctenopharyngodon idella* and observed decreased level of cholesterol. Virk and Sharma [35] studied biochemical changes induced by nickel and chromium in the liver of *Cyprinus carpio* and observed a significant diminution in the cholesterol content of the liver.

Triglyceride is the storage form of fats and major resources of oils and fat, which are flowing into the blood. The decrease in the quantity of cholesterol may be connected to its utilization in the manufacture of cortisol arising from stress created by the toxin atrazine [29]. The reduction of triglyceride volumes in blood plasma at high concentrations of the toxin atrazine could be due to the imbalance created by the higher concentrations of the toxin, affecting the digestive system, liver and related enzymes as well as hormonal and natural metabolic imbalance in fish studied.

In conformity with present study cholesterol, triglyceride, high density lipid and low density lipid have been discovered to be declined after famous treatment (200, 400, 800rpm) at different time interval (1, 7, 14, 21 and 28 days). It may be caused by utilization of cholesterol and other lipid fraction of treated fish to counteract toxic stress and stabilized the molecules of toxicants [36]. Further, this may be attributed to hindrance in lipid metabolism [36]. In accordance with the present finding, the similar decreased lipid profile has been reported by Ghosh 1988 [37] who reported the decline concentration of cholesterol in *Channa punctatus* against the chromium. Similar in *Clarias batrachus same* findings were observed by Khareet al [38] which was exposed to malathione. Furthermore, Sehgal and Goswami [39] Rani et al [40] Shankar and Kulkarani [41] observed the same findings in *Channa punctatus*, *Tilapamos sambuca*, *Notopterus notopterus* and *Cirrhinus mrigala* respectively. Therefore, all of the above studies were in accord with the present findings and therefore indicate the present resolutions.

Against stress, lipid profile concentration was also increased as indicated by different studies which are not in agreement with the present study. Intensity of hyper-lipemic state may reflect the degree of stress imposed on the animal under the influence of toxic reagents and environmental pollutants [42]. The increase of total plasma lipids may be due to the increase of lipid peroxides formation induced by the effect of butataf herbicide as previously reported by Mousa 2004 [43]. Otherwise, the destruction of the liver cells and other organs due to the effect of the butataf herbicide increase the levels of total lipids in the plasma [43-44].

5. CONCLUSION

Biochemical indices (lipid profile) of grass carp revealed that effects of atrazine herbicide. These fluctuations in biochemical indices can be considered as sensitive biomarkers i.e. for evaluating animal's health, especially in herbicides effecting region that causes stress to fish on exposure. The present results have proved the hazardous effects of the herbicide on the natural ecosystem with an alarming increase in pollution over the years. The current findings proved to clarify the risk of atrazine herbicide on lipid profile of grass carp fish. Decrease in lipid profile concentration [denoted by $P<0.05$ (significant), $P\leq0.01$ (highly significant) and $P\leq0.001$ (maximum highly significant)] was seen in all components of the lipid profile against various doses of atrazine, thus showing the contrary effects of atrazine on aquatic fauna.

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Research Article

Patterns in Anthelmintic Administration for Laying Hens in Blitar and Kediri District - Indonesia and the Opportunities of Drug Resistance

Lili Zalizar[1*], Wehandaka Pancapalaga[1], Dian Indratmi[1], and Yahya Jani[2]

[1]Department of Animal Science, University of Muhammadiyah Malang,
Jl. Raya Tlogomas No. 246 Malang, East Java, Indonesia 65144
[2]Departement of Biology and Environmental Science, Linnaeus University,
Stuvaregatan 4 SE-392 31 Kalmar, Sweden

Abstract: The purpose of this study was to determine the management of anthelmintic administration and the possibility of drug resistance in laying hens in Blitar and Kediri districts in Indonesia. This study consisted of two stages: first surveying 48 farmers in Blitar and 81 farmers in Kediri to find out how anthelmintic administration management included the frequency of anthelmintic administration for laying hens, types of anthelmintic, the habit of farmers using sustainably the same (> 3 yr) type of anthelmintic, determination of dosage, and use of herbal medicines in controlling worm disease. In the second stage, examine worm eggs at laying hens farms treated with worm medicine at intervals of 2 wk to 4 wk. The results showed that the majority of laying hens provide anthelmintic every 2 mo to 3 mo. In Blitar, the number of farmers who used the same worm medicine in more than 3 yr was 83.33 %. While in Kediri the number reached 97.53 %. The number of farmers who determined anthelmintic doses based on chicken body weight was 95.84 % (Blitar) and 90.12 % (Kediri). The administration of the same type of anthelmintic for more than three consecutive years and the calculation of anthelmintic doses based on the average body weight is thought to have an influence on the occurrence of drug resistance. It is seen that even though chickens were treated with anthelmintic for only 2 wk to 4 wk, worm eggs were found in fecal examination.

Keywords: Anthelmintic, Behaviour farmer, Drug administration, Herbal medicine, Immuno modullator, Worm disease.

1. INTRODUCTION

Food security is an important part of the right to food as well as one of the main pillars of human rights. It is therefore important that food security must be realized from the level of households, villages, districts and even the national level. Farmers have a strategic position in food security, as food producer, yet becoming the largest consumer group. Health is one of the factors that influence the improvement of chicken productivity. One of the diseases that often threaten the health of poultry farm infectious diseases due to worm infection. The health benefits of anthelmintic are very numerous. Anthelmintic administration is not only able to reduce the number of worm larvae but also increase the body weight of laying hens [1, 2]. Therefore, it is not surprising that many farmers uses anthelmintic.

During the past 30 yr the usage of factory-made anthelmintic was a common thing to do at the ranch not only in Indonesia but also worldwide. However administration of the same type of anthelmintic over a long period of time could trigger a drug resistance, so that the effect would become less effective. The use of less effective anthelmintic will be detrimental to farmers. The drug will not be able to kill the all the worms, since remaining worms that are still alive and will cause stunted growth and health of the chickens eventhough farmers have administer anthelmintic. Therefore, a series of research has been conducted about type of worms in poultry farm and pattern of anthelmintic administration on the farm and to assess their tendency to have drug resistance. Locations of research were limited in Blitar and Kediri, East

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* Corresponding Author: Lili Zalizar; lilizalizar62@gmail.com

Java province, which were considered as the center of chicken farms in Indonesia.

2. MATERIALS AND METHODS

This two-stage study was performed in Blitar and Kediri, East Java, Indonesia. The initial step was gathering information from 48 farmers in Blitar and 81 farmer laying hens in Kediri. This process was conducted by direct interview using a questionnaire. Then, result analysis of the questionnaire was compared with the worm eggs examination, taken from the feces of laying hens.

The method used in the first stage is a survey on the poultry farm that were using anthelmintic for at least three consecutive year. The questions proposed in the questionnaire regarding the type of worm that was often found, the type of anthelmintic used and management in anthelmintic administration (the frequency of drug administration, type of anthelmintic, farmer behavior in using the same type of an anthelmintic, the method of determining the dose and the use of herbal anthelmintic). Method on the second stage was conducting survey on a poultry farm that has been known to administer the same type of anthelmintic continuously for more than 3 yr. On the poultry farm, the stool samples were collected to calculate the number of worm eggs found in the feces. Sampling was repeated three times every 3 wk. The prevalence of worm infections are the number of animals infected with the worm (with the discovery of worm eggs) compared to the total laying hen sampled. The degree of infection is the number of worm eggs were found per gram of feces examined. The number of eggs g^{-1} of feces was calculated using the modified McMaster method with a sensitivity of 100 worm eggs g^{-1} of feces. Data were analyzed with descriptive methods.

3. RESULTS AND DISCUSSION

3.1. Type of Worms Found in the Poultry Farm

Most farmers in the district of Blitar and Kediri argued, Cestode was the type of worm that was most often found in poultry farm with a percentage of 70.83 % and 66.67 %. Cestode or tapeworms are type of worm whose life cycle requires intermediate host. Intermediate host for cestode are ants, flies and beetles rice. Those intermediate hosts are easily founded at chicken farm that has poor sanitation. This research was conducted at the local farm and generally managed with less attention to cage sanitation and environmental sanitation.

Environmental conditions affected the frequency of worm infections. Temperature of Blitar and Kediri district were 23 °C to 31 °C and 24 °C to 32 °C whereas humidity ranged between 70 % to 90 % and 65 % to 100 % [3]. This condition can support the growth of helminth's eggs and larvae in nature.

According to farmers, another worm which often attacked the laying hens was roundworm or including nematodes class. Nematode worm which is commonly found in the small intestine of laying hens is *Ascaridia galli* [4]. The worm's life cycle is very simple because it does not require intermediate host. Embryonated eggs come out from the chicken feces will hatch and grow up in the small intestine when ingested by other chicken. Given the direct life cycle, the opportunity to found of these worms in chicken farms is large.

3.2. Pattern in Anthelmintic Administration Management

Anthelmintic administration management patterns can affect the success of the control of worm infections in poultry farm. Anthelmintic administration management include the frequency of drug administration, type of anthelmintic, farmer behavior in using the same type of an anthelmintic, the method of determining the dose and the use of herbal anthelmintic

3.2.1. The Frequency of Anthelmintic Administration at Poultry Farm

In anthelmintic administration, farmers have different behaviors. Most farmers of laying hens in Blitar and Kediri give anthelmintic drug every 3 mo (43.41 %) and 2 mo (37.21 %). In addition, some farmers give anthelmintic drug every 6 mo (5.43 %); 4 mo (3.88 %); 1 mo (3.10 %). There are only 0.78 % of farmers who answered 5 mo, 7 mo and 8 mo. It can be concluded that the frequency of anthelmintic administration is every 2 mo to 3 mo.

Anthelmintic administration should be tailored to the degree of worm infections. The degree of

infection was the number of eggs/larvae per adult that worms were found in the body of livestock. Unfortunately, as much as 92 % of laying hens farmers in Blitar and 87 % in Kediri never performed laboratory tests to find out if their animal infected with worms before treatment. Some other farmers administer anthelmintic drug after worm was found in the chicken digestive tract during inspection held by health officials. The lack of laboratory facility was the main reason for farmers not to perform test to know the degree of infection. In fact, the price of tools and materials used for the examination of the degree of infection of worms were considered not expensive.

If the numbers of worm eggs are high, anthelmintic should be given to eradicate the worm and prevent interference in productivity or death. However, if the number is still very low, anthelmintic administration is not require because to maintance immunity of the poultry against parasite antigen. Continuous anthelmintic administration without any indication could lead to worm resistance toward those anthelmintic.

Farmers in Blitar and Kediri give anthelmintic every 2 mo to 3 mo. Anthelmintic administration aims to break the life cycle of worm that lasts ranges between 1 mo to 3 mo. But, it is more preferable to examine the degree of infection before administering anthelmintic to ascertain whether anthelmintic is needed.

3.2.2. The Type of Anthelmintic Used at Laying Hen Poultry Farm

Number of farmers of laying hens in Kediri mostly used Benzilmidazol group and Levamisol reached 48.15 %. Types of anthelmintic from Benzimidazole groups like Albendazole, and Fenbendazol. Benzimidazole is an effective anthelmintic to eradicate worms of the class Nematoda, Cestoda , and Trematoda. On the other hand, farmers in Blitar were most likely to use piperazine (64.42 %). Piperazine was considered effective for Nematode worms from the Ascarididae family (for example *A. galli*). However, when considering the majority of the farm in Blitar stricken with Cestode then treatment with piperazine will be less effective.

At the poultry farm in the district of Kediri most farmers used Benzimidazole group and levamisol. On the other livestock such as sheep, there were already many reports of some types of worms that develop resistance to this kind of anthelmintic. According to Garcia et al. [4], there has been a resistance to some anthelmintic in worms that attacked sheep in Colombia. It was seen from the efficacy of anthelmintic such as albendazole only between 0 % to 55 %; fenbendazole 51.40 % to 76.6 %; and levamisole: 0 % to 78.1 % [4]. This result was the first discovery of the existence of multi-resistant against anthelmintic in Colombia. In Tamil Nadu, India, there has been reported the emergence of anthelmintic resistance on sheep farm [5]. The researchers reported the existence of the resistance of worms to Benzimidazole group and levamisole. There was possibility that worm contained in laying hens already developing nature of anthelmintic resistant regarding the pattern of anthelmintic administration in the two districts that used the same type for more than 3 yr [5].

3.2.3. Farmers Behavior in using the Same Type of an Anthelmintic in a Long Term (> 3 yr) on an Ongoing Basis

Most farmers in Blitar and Kediri admitted that they have been using the similar anthelmintic for more than three years. In Blitar, the number of farmers who used the same anthelmintic in more than 3 years is as much as 83.33 %. While in Kediri the number reached 97.53 %. Administration of the same type of anthelmintic for a long period can lead to anthelmintic resistance [6–8]. Long term administration of the same type of anthelmintic was already accustomed and considered more effective than herbal drug.

3.2.4. Determination of Anthelmintic Dose

In Blitar and Kediri, the number of farmers who determined anthelmintic dose based on chicken's body weight was 95.84 % (Blitar) and 90.12 % (Kediri). There was no farmer who determines anthelmintic dose based on the biggest chicken's body weight in Kediri. Meanwhile 2.08 % of farmers in Blitar determined anthelmintic dose based on the biggest chicken's weight.

Determination of anthelmintic dose should be based on every chicken body weight individually. If anthelmintic dose was determined base upon average body weight, then chicken that has body

weight higher than the average chicken body weight will be underdosed. This condition caused only highly sensitive worms will be killed while others will survive and develop anthelmintic resistance and pass the resistance properties to the offspring [8].Most farmers considered that determination of anthelmintic based on individual body weight was not practical and requires automatic weighing instrument [8].

3.2.5. The use of Herbal Medicine in Controlling Worm Diseases

Most farmers having egg laying hens in Blitar and Kediri never use herbal medicine in controlling worm infection. The 75 % egg of laying hens in Blitar and 83.95 % in Kediri answered that they never used herbal medicine to overcome worm disease. Only 8.33 % of farmers in Blitar and 9.88 % in Kediri have used herbal remedies. Meanwhile 8.33 % farmers in Blitar and 6.17 % farmers in Kediri did not answer.

Generally farmers used garlic (*Allium sativum* L.), ginger (*Zingiber officinale* Roscoe.) and turmeric (*Curcuma longa* L.) to overcome the parasitic worms. Some herbal medicines have been widely studied for the ability to kill the worms, like *Citrus aurantifolia* (Christm.) Swingle peel extract [9], pomegranate (*Punica granatum* L.) peel extract [10] and paw paw (*Carica papaya* L.) seeds [11] Herbal also have potensial as Immuno modullator [12]. The attitude of farmers who rarely seek information about the efficacy of herbal medicine in disease treatment led to wrong perception. Most farmers did not use herbal medicines since they did not understand the benefit. While the reason for the farmers who use herbal medicine was because there were no side effects and thus more secure. In addition, some farmers said that by using herbal medicines they can save more costs. Herbal medicine, which was available around the farm, also makes it easier to be found and available to be used at any time.

3.3. The Impact of Anthelmintic Resistance Development in Poultry Farm that uses the Same Type of Anthelmintic for More than 3 years

Worm infection was found in laying hen that treated (2 wk to 4 wk before worm eggs examination) with similar anthelmintic continuously for more than three or more years (Table 1). This indicated the likelihood of the development of worm resistance against anthelmintic given.

In Sweden fenbendazol is considered effective in poultry, but surprisingly the risk of reinfection after administration of fenbendazol is still high [13]. The decline in the number of worm eggs after anthelmintic administration was only temporary, ranged between 2 wk to 4 wk post administration. It was due to suboptimal anthelmintic administration or anthelmintic resistance of worm larvae that are still present in the tissue. The larvae survive and develop into adult worms then produce eggs that were released with feces.

Administration of the same anthelmintic for three or more consecutive year and calculating anthelmintic dose based on the average body weight is thought to have influence on the occurrence of drug resistance. It can be seen from the result of laying hens worm eggs observations that was collected from the fecal examination (Tabe 1). The observation was undertaken in laying hens that was recently treated with anthelmintic albendazole (2 wk to 4 wk before the observation) by searching for the present of worm eggs in feces. In Blitar, from 100 laying hens that were examined, worm eggs was positively found in seven laying hens (worm disease prevalence reached 7 %). While in Kediri, from 120 laying hens that were examined, worm eggs was positively foung in 17 laying hens (14.17 %). Type of eggs worm that found were *Ascaridia galli* and *Heterakhis gallinarum*.

Table 1. Prevalence of layer infected with worms (%) after being treated with anthelmintic

No	Region	Number of Laying Hens Examined	Worm Eggs*		Prevalence (%)
			Positive	Negative	
1	Blitar	100	7*	93	7
2	Kediri	120	17*	103	14.17

*worm eggs examination was held 2 wk to 4 wk post anthelmintic administration.

Those facts showed that it was probable that worm resistance against anthelmintic was developing because worm eggs are still present from the fecal examination. Given the nature of drug resistance which can be transmitted by the worm to its offspring, the opportunity of increasing number of worm resistance to anthelmintic was high so that the treatment became less beneficial.

4. CONCLUSION

The results showed that most egg laying hens were given anthelmintic for 2 mo to 3 mo, anthelmintic types that are often used are Benzimidazole Group, Levamisole (in Kediri) and Piperazine (in Blitar). In Blitar, the number of farmers who used the same anthelmintic in more than 3 yr is 83.33 %. While in Kediri the number reached to 97.53 %. The number of farmers who determined anthelmintic dose based on chicken's body weight was 95.84 % (Blitar) and 90.12 % (Kediri). Most farmers having egg laying hens in Blitar and Kediri never use herbal medicine in controlling worm infection. Administration used the same anthelmintic for three or more consecutive years and calculated anthelmintic dose based effect on the average body weight is thought to influence the occurrence of drug resistance. According to farmers, the most common type of worm in farm are tapeworm (Cestode) and roundworms (Nematode). But from the results of feces examination only Nematodes *Ascaridia galli* and *Heterakhis gallinarum* were observed.

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Research Article

Physicochemical Properties of Soil as Affected by Land Use Change in a Tropical Forest Ecosystem of Northeastern Bangladesh

Md. Habibur Rahman[1,2*], and Md. Bahauddin[3]

[1]Laboratory of Tropical Forest Resources & Environments, Division of Forest & Biomaterials Science, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
[2]Bangladesh Institute of Social Research Trust, Lalmatia, Dhaka, Bangladesh
[3]Department of Narcotics, District Narcotics Control Office, Jashore, Bangladesh

Abstract: Some selected soil physicochemical properties such as nitrogen (N), phosphorus (P), potassium (K), soil organic carbon (SOC), carbon to nitrogen ratio (C: N), and soil organic matter (SOM) with soil p^H have been evaluated from *Shorea robusta* and *Dipterocarpus turbinatus* plantations, and a deforested site, and compared to the surface soil (0-10 cm) and sub-surface soil (10-30 cm) at Tilagarh Eco-park of northeastern Bangladesh. Total 90 soil samples were collected from 20m × 20m plot in two soil depths among 30 from each land use and 15 from each soil depth. The data were analysed statistically through one-way ANOVA test and compared by using DMRT at $p < 0.05$. The average of the mean value of soil p^H (5.33 ± 0.058) was significantly higher in *S. robusta* plantation (5.6 ± 0.096) than *D. turbinatus* plantation (5.25 ± 0.042). Available K content was significantly higher in *S. robusta* plantation (62.56 ± 0.004 mg kg^{-1}) than *D. turbinatus* plantation (54.74±0.016 mg kg^{-1}). There was a significant difference between P content in *D. turbinatus* (4530 ± 0.319 mg kg^{-1}) and *S. robusta* (4210 ± 0.088 mg kg^{-1}) plantations. Total N content was slightly higher in *S. robusta* plantation (0.11 ± 0.01%) than the *D. turbinatus* plantation (0.10 ± 0.00%). The mean value of SOM concentration was higher in *S. robusta* plantation (1.83 ± 0.180%) than in *D. turbinatus* plantation (1.72 ± 0.026%), and SOC concentration was found higher in the *D. turbinatus* plantation (2.58 ± 0.15%) than in *S. robusta* plantation (2.35 ± 0.21%). Finally, the study showed that there was a significant difference in the mean values of physicochemical properties according to two soil depths and three land uses.

Keywords: Physicochemical Properties, Plantation, Deforestation, Soil p^H, Land Use Change, *Shorea robusta, Dipterocarpus turbinatus.*

1. INTRODUCTION

Plants play the most significant role in developing the macro and micronutrient properties of soil due to its influence on local climate, nutrient or ecological cycling, moisture, microorganisms, soil erosion and hydrological cycle [1-2]. Constant deforestation and forest degradation are deteriorating the forest resources with a damage of habitat, loss of biodiversity, risk of species extinction, reducing soil productivity through erosion and desertification, and reducing natural seed germination and regeneration survival [3]. Inappropriate agricultural practices, burning, overgrazing, overexploitation of forest resources, exotic species invasion or by a combination of factors in the tropical forests have a great effect on physical, chemical and biological properties of the soil resources [4-5]. Land use change is the most important drivers affecting plant communities, species composition and structure, biodiversity and functioning of terrestrial ecosystems, soil properties, and microorganisms [6-7]. Instead, global plantation forests which are an important element of land use change are increasingly significant in the world's future timber supply and in most area's plantations are established on disturbed and degraded soils [8]. Plantations may play a major role in increasing soil fertility and can differ in their influence (between native and exotic species plantation, N_2-fixing and non-

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*Corresponding Author: Md. Habibur Rahman; habibmdr@gmail.com

N_2-fixing species) on soil physical and chemical changes [9]. These changes are more biologically and chemically than physically [10-11] which leads to the decline of physical, chemical and biological potential of soil and endanger local biodiversity [12].

Soil physicochemical properties such as nitrogen (N), phosphorus (P), potassium (K), soil organic matter (SOM), and soil organic carbon (SOC) are essential elements and determinants of plant growth [13-14]. Likewise, soil reaction or pH is a most important chemical property of soil and one of the determining factors in the plant nutrient availability in the soil [15]. The p^H range of an ideal soil for plant growth is slightly acidic (p^H 6.5) to slightly alkaline (p^H 7.5) [16]. On the other hand, a number of plant nutrients are unavailable at extremely acidic or extremely alkaline soils due to the different reactions in the soil which fix the nutrients and transform them to the state that is unavailable for the plants [17]. Hence, to know the ideal soil for plants, it is important to understand soil chemistry and interacting factors that affect soil p^H, and the effects of p^H on nutrient availability [18].

Land use change and forest management practices directly alter soil biodiversity and soil quality that in turn affecting forest ecosystem functions and productivity. Soil P and N are the key nutrients for tree growth and there is a close relationship between them with SOC cycling [19], which have a potential role to mitigate the effects of global climate change [20]. Likewise, the imbalance of soil physicochemical properties is expectedly affected and control the ecosystem productivity, processes and carbon-storage capacity of tropical forests ecosystems including Amazonian, Neotropical and Bornean forests [21-25]. Consequently, it is obligatory to enhance current knowledge on the availability of soil physicochemical to assess the forest ecosystem productivity, as well as their role in the global carbon cycle [20, 26].

Bangladesh is a tropical South Asian country has an area of 14.76 million hectares of which only 2.6 million hectares is forestland equivalent to 17.62% of the country's land area, compared to the minimum requirement of 25% forest cover of a country. Out of which, 1.58 million hectares or 60.77% are managed by Bangladesh Forest Department (here *BFD*); 84% are natural forests (primary and secondary) and rest 16% are plantation forests [27]. Bangladesh has tropical evergreen and semi-evergreen hill forest, tropical moist deciduous Sal (*Shorea robusta* Gaertn. f.) forest, natural mangrove forest in Sundarbans, coastal mangrove plantation, and fresh water swamp forest. The hill forests cover 38.2% and characterized by mixed evergreen and semi-evergreen plant communities of trees with diverse herbs, shrubs, rattans and bamboos [28-29]. Historically forest resources continuously depleted by overexploitation of resources, illicit felling, fuelwood collection, grazing, agricultural and settlement expansion inside the forest boundary [30-32]. It was estimated that forest cover of the country has been declining at the rate of 2,600 hectares per year [33]. To combat this depletion, BFD declared reserved forest, protected area and involved community in participatory social forestry plantation and the latest (2003 to 2018) implemented co-management projects in 18 out of 34 protected areas [34]. At the same time, to meet the country demand of timber and fuelwood, forest plantations were raised with exotic timber species (like *Acacia* spp.) followed by fruit-bearing, medicinal and fuelwood species [35].

Mixed tropical forests in Bangladesh covers the east, northeastern and southeastern hill regions with an area of 18,079 sq km. The hills comprise the Tipam-Surma (50%), Dupi Tila (40%) and alluvial soils (10%). The hill soils (brown hill soils) are moderately fertile in respect of their physicochemical properties and mineral nutrient content [36]. Hossain et al [37] and Rahman et al [38] analyzed soil physical properties in the northeastern tropical forest ecosystems however, no research works have been done yet to evaluate the relation of soil physicochemical properties in the plantations and deforested sites. Soil physicochemical information is crucial for understanding the ecosystem processes, conservation efforts and sustainable forest management in tropical forest ecosystems. Moreover, there is a lack of information on how forest plantations affect soil pH and physicochemical properties comparing the degraded forest soils. Taking this into consideration, the objective of this study was to analyse some selected

soil physicochemical properties such as available P and K, total N, SOM, SOC, and carbon to nitrogen ratio (C: N) and soil p^H in two plantation sites (*S. robusta* and *Dipterocarpus turbinatus* Gaertn) and a deforested site in a tropical forest ecosystem namely Tilagarh Eco-park (TGEP) of northeastern Bangladesh. The study also assesses the difference among the soil samples of two plantation sites and deforested site and between surface soil and sub-surface soil within three land uses.

2. MATERIALS AND METHODS

2.1. Study Site

The Tilagarh Eco-park is located in Sylhet Sadar Upazila (sub-district) of Sylhet District under North Sylhet Range-1 of the Sylhet Forest Division. Geographically, the park lies between 23°55′ and 25°02′ N latitude and 90°55′ and 92°30′ E longitude (Fig. 1). BFD has declared this as an eco-park in 2006 (with an area of 45.34 ha) from the Tilagarh Reserve Forest [38] with the objective of preservation and development of almost extinct and rare species of flora, protection and development of existing flora and fauna, breeding and development of local species of flora through intestine management, expansion of planned eco-tourism, and the creation of opportunity for study and research [39]. Adjacent to the park, there is a tea estate named *'Laccatora Tea Estate'*, which is the oldest tea estate in the Indian sub-continent.

TGEP is a tropical semi-evergreen dense forest with more than 90 tree species of 13 families among *S. Robusta* & *D. turbinatus* are the dominant tree species. Other common natural and planted tree species include *Michelia champaca* Linn., *Artocarpus chaplasha* Roxb., *Lagerstroemia speciosa* (L.) Pers., *Mimusops elengi* L., *Mesua ferrea* L., *Barringtonia acutangula* (L.) Gaertn., *Delonix regia* (Hook.) Raf., *Bombax ceiba* L., *Aquilaria agallocha* Roxb., *Mangifera indica* L., *Zanthoxylum rhetsa* (Roxb.) DC., *Artocarpus heterophyllus* Lamk., *Elaeocarpus floribundus* Blume, *Casuarina littoralis* (Salisb.), *Albizia lebbeck* (L.) Benth. & Hook., *Senna siamea* (Lamk.) Irwin & Barneby, *Camellia sinensis* (L.) O. Kuntze with different types of naturally grown rattans e.g., *Calamus tenuis*. *S. robusta* plantation was established in 1990-91 which covers nearly 60% of the total vegetation cover. About 206 species of fauna has been recorded in the park which include seven amphibians, 28 mammals, 30 reptiles, and 141 birds.

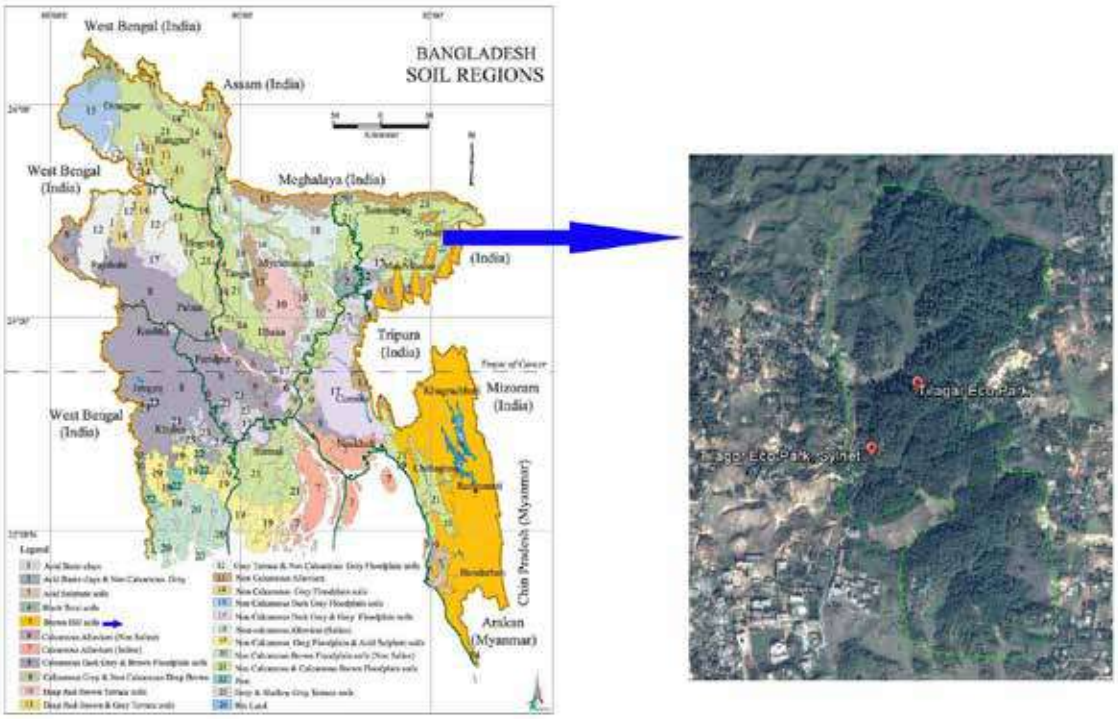

Fig. 1. Map of Bangladesh soil regions indicating the location of Tilagarh Eco-park in northeastern Bangladesh

Climatic is hot and humid summer and a relatively cool winter. The highest average temperature of the park is 31.6˚C between August and October, and the lowest average temperature is 7˚C between January and February. The park is located in a moist tropical climatic zone with average annual rainfall of about 3,937 mm, where nearly 80% occur between May and September [38]. A tide of stream flows across the park and there are having several hillocks (locally called *tilla*), ranging from 10-50 m height. These hillocks abound with many naturally grown trees. The soil of hillocks varies from clay loam to pale brown (acidic) clay loam, which are moderately fertile with low soil p^H, ranges from 5 to 7.5. Red sandy clay contains manganiferous iron ore. However, soil erosion is common during monsoon, and hillocks soil contains a lot of minerals and nutrients due to upland leaching supporting more growth.

2.2. Research Methods

For the study, soil samples were collected from three land use sites among the two was dominant plantation sites, *i.e.,* one is *S. robusta* plantation and other is *D. turbinatus* plantation, another from the deforested site. Soil samples were collected by using a soil core from surface soil (0-10 cm depth) and sub-surface soil (10-30 cm depth) and properly labelled in air tied polybags to prevent the loss of soil moisture. Total 90 soil samples were collected from 20m × 20m square plot among 30 samples were from each land use (two plantation sites and a deforested site) and15 samples from each soil depth (0-10 cm and 10-30 cm). After that, a composite soil sample had been made by mixing every five soil samples, and a total of 18 composite soil samples was prepared and stored for further laboratory analysis.

2.2.1. Soil Sample Preparation

In the laboratory, at first collected soil samples were sieved through a 10 mm mesh sieve to remove visible gravels, coarse roots, and small stones, and then passed through another 2 mm sieve for an oven dry at 105 ºC for eight hours till constant weight.

2.2.2. Determination of Soil pH & Physicochemical Properties

Soil reaction (p^H) was determined by using a digital portable waterproof p^H meter HANNA HI 9210N ATC (range 0.00 to 14.00 p^H; accuracy (@20°C) ±0.02 pH / ±0.5°C). A mixed solution of soil: distilled water (1:2) was used to measure the soil p^H (www.hannainst.com). Soil physicochemical properties such as total N (%) was determined by the longer than a century year old Micro-Kjeldahl digestion, distillation and titration method [40]; available P (mg kg^{-1}) was determined by using the Bray and Kurtz method [41]; available K (mg kg^{-1}) was determined by the Ammonium acetate method [42]; total SOC (%) was determined by using the loss of ignition method [43], and SOM content was then calculated by multiplying the percent of SOC by a factor of 1.724 [44]. This follows the standard practice that SOM is composed of 58% carbon. On the other hand, the determination of C: N ratio was calculated through the ratio of SOC (%) and total N (%) or simply, SOC/total N.

2.2.3. Data Analysis

The data were analysed statistically through one-way ANOVA test to know the significant difference among the soil samples of three different land uses by using SPSS 17.0. The ANOVA results were then compared by using Duncan's Multiple Range Test (DMRT) at $p<0.05$ [45] and arranged systematically.

3. RESULTS AND DISCUSSION

3.1. Soil p^H

Soil p^H affects the number of available nutrients and chemicals that are soluble in soil water. Precipitation is considered a great influencing factor to determine soil p^H because rain water leaves elements in the soil that produce acid and carries and solubilized nutrients in soils. The present study suggests that the soil under *D. turbinatus* plantation and deforested site were subjected to erosion that allowed to leach the base-cations from two sites. Deforested site and *D. turbinatus* plantation were more susceptible to erosion than *S. robusta* plantation. Even, it may be due to the acid neutralization characteristics of the components contained in the leaf litters of the *S. robusta*. Changes in land use and deforestation might have a significant effect on the alteration (increase or decrease) of soil acidity [46]. Comparable, SOM through litter decomposition decrease the soil p^H and increased soil acidity in the hill forests of Bangladesh [47] which is seen more in the natural forest than that of plantation forest

[48].

The present study revealed that there was a significant difference in soil p^H in three land use regardless of surface and sub-surface soil. The average of the mean value of soil p^H (5.3 ± 0.058) was significantly higher ($p < 0.05$) in *S. robusta* plantation (5.6 ± 0.096) than that in *D. turbinatus* plantation (5.3 ± 0.042) (Table 1). Hence, it can be considered that the soil of this zone is mostly acidic in nature and not the most nutritious soil for all kinds of plants. That means the trend of acidity decreases with the conversion of deforested land into *S. robusta* plantation. The results showed Similarly, Biswas et al [49], Gafur et al [50], and Osman et al [51] also recorded higher soil p^H in shifting cultivation lands compared to forested lands in this region. Akhtaruzzaman et al [52-53] recorded the higher p^H values at both surface and sub-surface soils of the cultivated site as compared to planted forest and barren land. Haque and Karmakar [47] also estimated that matured mixed plantation forests showed lower p^H than younger plantation forests.

3.2. Available Potassium in Soil

Available K in soil is a vital nutrient for plants and

Table 1. Soil p^H at two soil depths in two plantation sites and a deforested site.

Land use and land cover	Soil depth (cm)	Mean value	Avg. of mean
S. robusta plantation	0-10	5.8 ± 0.057^{d}	5.6 ± 0.096
	10-30	5.4 ± 0.057^{c}	
D. turbinatus plantation	0-10	5.3 ± 0.057^{bc}	5.3 ± 0.042
	10-30	5.2 ± 0.057^{ab}	
Deforested site	0-10	5.2 ± 0.057^{ab}	5.2 ± 0.042
	10-30	5.1 ± 0.057^{a}	
Total			**5.3 ± 0.058**

Note: ±: Standard deviation; Values with different lowercase (a, b, c….) letters are significantly different in the same soil layers at three land use change ($p < 0.05$) according to DMRT.

that the soil under *D. turbinatus* plantation and deforested site were more acidic than the soil of under *S. robusta* plantation. Here, SOM through continuous litter fall mostly influences the high soil p^H in the surface soil. Likewise, N and K are fewer directly affected by soil p^H, but P is directly affected by soil p^H [16].

Roy et al [31] recorded more acidic soil in banana-based agroforestry soil than the *S. robusta* forest soil due to the application of various fertilizers, growth hormones and pesticides for better production in the agroforestry field. Zaman et al [46] found that soil p^H decreased with depth and p^H values was significantly higher in forested sites in comparison to the deforested sites. Haque et al [48] found soil p^H is significantly higher in the deforested land than adjacent forest soil in an upland watershed of Bangladesh. Biswas et al [49] recorded lower p^H in the soil of Chittagong Hill Tracts due to continuous shifting cultivation farming systems but slightly higher from the adjacent natural forests.

most forest soils have substantial quantities of K in solution which plays many important regulatory roles in plants growth and development [54]. The present study found that the average of the mean value of available K in the soil was 54.74 ± 0.007 mg kg^{-1} which was significant at $p<0.05$. The lower value of K was recorded in the soil of deforested site (46.92 ± 0.006 mg kg^{-1}) where highest was recorded in the soil of *S. robusta* plantation (62.56 ± 0.004 mg kg^{-1}) (Table 2). The inconsistency in the availability of K in the soil of three land uses due to the less abundance of vegetation on the deforested site and presence of comparatively more litter fall under the *S. robusta* plantation.

In respect of surface soil, statistically no significant variation was found among the available K in *S. robusta* and *D. turbinatus* plantations and a deforested site. But in respect of sub-surface soil, statistically significant variation (4.9% decrease) was found between the values of K in two plantation sites due to the accumulation of more K

Table 2. Available potassium and phosphorous at two soil depths in two plantation sites and a deforested site

		Potassium (mg kg^{-1})		Phosphorous (mg kg^{-1})	
Land use and land cover	**Soil depth (cm)**	**Mean value**	**Avg. of mean**	**Mean value**	**Avg. of mean**
S. robusta plantation	0-10	58.65 ± 0.006^{cd}	62.56 ± 0.004	4410 ± 0.02^{d}	4210 ± 0.088
	10-30	62.56 ± 0.006^{d}		4010 ± 0.02^{b}	
D. turbinatus plantation	0-10	66.47 ± 0.011^{d}	54.74 ± 0.016	5240 ± 0.02^{e}	4530 ± 0.319
	10-30	39.10 ± 0.006^{a}		3810 ± 0.00^{a}	
Deforested site	0-10	50.83 ± 0.006b^{c}	46.92 ± 0.006	4300 ± 0.02^{c}	4040 ± 0.117
	10-30	43.01 ± 0.006^{ab}		3780 ± 0.01^{a}	
Total			**54.74 ± 0.007**		**4300 ± 0.121**

Note: ±: Standard deviation; Values with different lowercase (a, b, c….) letters are significantly different in the same soil layers at three land use change ($p<0.05$) according to DMRT.

in the surface soil and subsequent leaching of them from the surface soil to the sub-surface soil of *S. robusta* plantation. The survey results revealed that the value of available K decreased from surface to sub-surface soil in *D. turbinatus* plantation and deforested site (Table 2).

Akhtaruzzaman et al [53] found the higher value of available K in the cultivated soil than plantation forest soils and barren land soils as cultivated soil received more K from applying ash during soil management for cultivation. Similarly, Roy et al [31] also estimated higher K in agroforestry soil than the soil of *S. robusta* forest. Instead, Zaman et al [46] and Biswas et al [49] recorded higher K in the soil of forested sites in compared to deforested sites. Similarly, Biswas et al [49], Osman et al [51] and Biswas et al [55] estimated higher value of available K in the soil of Chittagong Hill Tracts of Bangladesh which has no history of shifting cultivation practices. Similar to the present study, Akhtaruzzaman et al [52-53] and Akbar et al [56] found that total available K in the soil decreased with increasing soil depths.

3.3. Available Phosphorus in Soil

Available P in soil is the most common physicochemical limiting plant growth in terrestrial ecosystems [57]. P is vital for the plant's life specifically for the storage and reproduction of plant genetic material and for energy-related processes called photo-phosphorylation [58]. Scholars reported that the soil of tropical and subtropical regions have a P deficiency and contains a lower amount of available P [59] that could be lessened these forest ecosystem responses to the increasing global carbon dioxide concentrations [60]. Survey data revealed that *D. turbinatus* plantation soil contained the highest average of the mean value of available P (4530 ± 0.319 mg kg^{-1}) followed by *S. robusta* (4210 ± 0.088 mg kg^{-1}) plantation soil and the soil of deforested site (4040 ± 0.1168 mg kg^{-1}) respectively at $p<0.05$ level of significance (Table 2). The results suggested that the rate of nutrient loss in the guise of soil erosion was higher on deforested land and *S. robusta* plantation than the *D. turbinatus* plantation. On the other hand, as the leaves of *D. turbinatus* plant is highly flammable, so that higher P value was found in the soil under *D. turbinatus* plantation.

The present study showed that the P value in soil varied for land use change in respect of soil depths. It was found that surface soil contains more P than the sub-surface soil. At surface soil, the mean value of P was higher in *D. turbinatus* plantation (5240 ± 0.02 mg kg^{-1}) and less in the deforested land (4300 ± 0.02 mg kg^{-1}), with no statistically significant variation between the *S. robusta* and *D. turbinatus* plantations. Considering the sub-surface soil, the highest mean value of P was found in *S. robusta* plantation (4010 ± 0.02 mg kg^{-1}) and less in the deforested land (3780 ± 0.01 mg kg^{-1}) as well as significantly different from the three land uses (Table 2).

Similar to the present study findings, a number of studies e.g., Haque et al. [48], Akhtaruzzaman et al [52-53] and Akbar et al [56] reported that the hill soils in Bangladesh are poor in available P. This study revealed that the mean value of available P

Table 3. Total nitrogen and carbon to nitrogen ratio at two soil depths in two plantation sites and a deforested site

Land use and land cover	Soil depth (cm)	Total nitrogen (%)		Carbon to nitrogen ratio	
		Mean value	Avg. of mean	Mean value	Avg. of mean
S. robusta plantation	0-10	0.13 ± 0.01^{d}	0.11 ± 0.01	11.4 ± 0.02^{b}	17.95 ± 0.02
	10-30	0.08 ± 0.01^{b}		24.5 ± 0.03^{c}	
D. turbinatus plantation	0-10	0.10 ± 0.01^{c}	0.10 ± 0.00	14.3 ± 0.02^{bc}	19 ± 0.02
	10-30	0.10 ± 0.00^{c}		23.7 ± 0.03^{c}	
Deforested site	0-10	0.01 ± 0.00^{a}	0.03 ± 0.01	7.7 ± 0.01^{a}	9.3 ± 0.01
	10-30	0.05 ± 0.01^{b}		10.9 ± 0.02^{b}	
Total			**0.08 ± 0.01**		**15.42 ± 0.02**

Note: ±Standard deviation; Values with different lowercase (a, b, c….) letters are significantly different in the same soil layers at three land use change ($p<0.05$) according to DMRT.

was decreased with soil depths in three land uses which corresponding with the findings of Biswas et al [49] might be due to the lower amount of or SOM and higher fixation of P in sub-surface soil. On the other hand, Mia et al [61] estimated that the value of available P was higher in the soil of mixed forest stand than the pure forest and plantation forest stands.

3.4. Total Nitrogen in Soil

N is one of the major components of the atmosphere, and this atmospheric N is a source of soil N which is a key element of plant growth. SOM is a major pool for soil N content which is present in the form of nitrates. Forest soils contain large organic N pools which have a positive impact on the terrestrial carbon sequestration [62]. Study data revealed that the average of the mean value of total N content was slightly higher in *S. robusta* plantation soil (0.11 ± 0.01%) than the *D. turbinatus* plantation soil (0.10 ± 0.00%) compared to the soil of deforested site (0.03 ± 0.01%) in two soil depths at $p<0.05$ level of significance (Table 3). *S. robusta* plantation soil contained the higher mean value of total N (0.13 ±0 .01%) than the *D. turbinatus* plantation soil (0.10 ± 0.01%) at the surface soil. On the other hand, at sub-surface soil, the highest mean value of total N was found in the soil of *D. turbinatus* plantation (0.10 ± 0.00%) followed by *S. robusta* plantation soil (0.08 ± 0.01%) (Table 3).

The hill forest soil in Bangladesh has a lower content of total N which also supported by the present study. Similar to the study, Zaman et al [46] stated that due to the presence of litter and humus in the upper soil layer, this layer content higher amount of N which accelerated soil water holding capacity. Shaifullah et al [63] found that total soil N content was increased due to afforestation on a coast of Bangladesh. Roy et al [31] found more N content in agroforestry soil than the *S. robusta* forest soil. Similar to the present study findings, Haque et al [48], Akhtaruzzaman et al [52-53] and Akbar et al [56] found higher N content in the planted forest soil in comparison to barren and cultivated land soils. Mia et al [61] calculated the lower content of total N in pure forest stand soil compared to the plantation and mixed forests stands soils.

3.5. Soil Organic Matter

SOM is mainly derived from plant litter and humus and affects both physical and chemical properties of the soil and improve soil health [62]. SOM is supplying most of the nutrients held in the soil and affects the stabilization in soil aggregates, soil structure and porosity, increases water holding capacity, and increases the diversity and biological activity of soil microorganisms. Most soils contain 2 to 10% SOM [64]. However, much of the forestry research on the impacts of plantations on SOM concerns the plant OM component or biomass analysis, leaving out the soil-incorporated OM [65].

Table 4 shows the value of SOM in three land uses in the study area. Survey data revealed that surface soil contains a higher amount of SOM in three studied land use. The mean value of SOM was higher in *S. robusta* plantation soil (1.83 ± 0.180%) than in *D. turbinatus* plantation soil (1.72 ± 0.026%). It was found that surface soil contains more SOM

than the sub-surface soil. At surface soil, the value of SOM was found higher in *S. robusta* plantation (2.24 ± 0.011%) than the *D. turbinatus* plantation (1.77 ± 0.023%). In the sub-surface soil, the value of SOM was found higher in *D. turbinatus* plantation (1.67 ± 0.023%) than the *S. robusta* plantation (1.41 ± 0.011%). In case of soil depths, the average of the mean value of SOM varied in three land uses with the increased of soil depth (from the surface to sub-surface soil) which also significantly different at the $p<0.05$ level of significance. In case of the average of the mean value of SOM, the higher value was found in *S. robusta* plantation soil (1.83 ± 0.180) than the *D. turbinatus* plantation soil (1.72 ± 0.026) (Table 4 & Fig 2).

In the forested soil, it is common that the surface soil contains more SOM than the sub-surface soil [66]. On the other hand, soils of Bangladesh are generally low in SOM having less than 1.5% in most of the soils, and some soils have even less than 1% SOM [67]. Specifically, the soils of the northern and eastern hills are low in SOM and poor in general fertility [68]. Haque et al [48] recorded higher SOM content in forest soil compared to deforested soil. Alike, Roy et al [31] also estimated higher SOM in banana-based agroforestry soil than the *S. robusta* forest soil as the banana cultivation required frequent organic and chemical fertilizers, different pesticides and growth hormones for better plant growth and banana yield. All the above-mentioned findings agreed with the present study findings. Biswas et al [49] recorded lower content of SOM in the soils of Chittagong Hill Tracts due to continuous shifting cultivation and farming.

Table 4. Soil organic matter and soil organic carbon at two soil depths in two plantation sites and a deforested site

Land use and land cover	Soil depth (cm)	Soil organic matter (%)		Soil organic carbon (%)	
		Mean value	Avg. of mean	Mean value	Avg. of mean
S. robusta plantation	0-10	2.24 ± 0.011^{e}	1.83 ± 0.180	1.90 ± 0.19^{d}	2.35 ± 0.21
	10-30	1.41 ± 0.011^{c}		2.79 ± 0.23^{e}	
D. turbinatus plantation	0-10	1.77 ± 0.023^{de}	1.72 ± 0.026	1.69 ± 0.19^{c}	2.58 ± 0.15
	10-30	1.67 ± 0.023^{d}		3.47 ± 0.10^{f}	
Deforested site	0-10	0.31 ± 0.006^{b}	0.27 ± 0.189	0.32 ± 0.20^{a}	0.56 ± 0.15
	10-30	0.23 ± 0.006^{a}		0.85 ± 0.11^{b}	
Total			**1.27 ± 1.780**		**1.83 ± 0.17**

Note: ±: Standard deviation; Values with different lowercase (a, b, c….) letters are significantly different in the same soil layers at three land use change ($p<0.05$) according to DMRT.

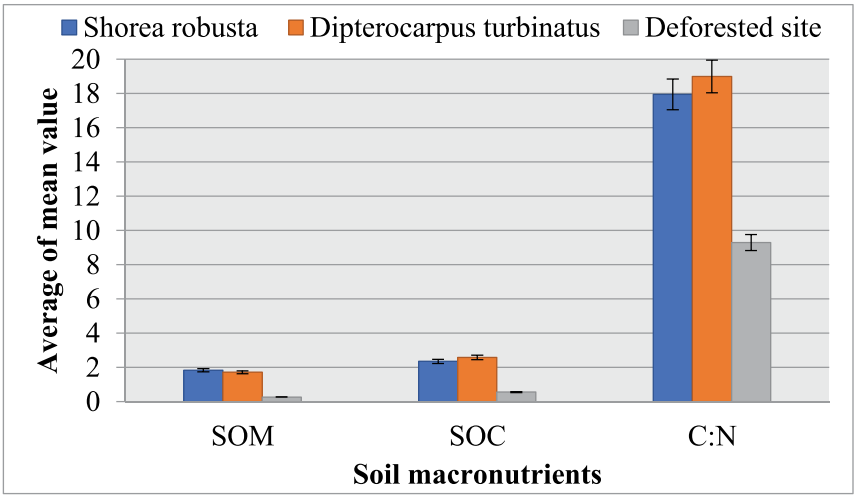

Fig. 2. Average of the mean value of soil organic matter (SOM, %), soil organic carbon (SOC, %) and carbon to nitrogen ratio (C: N)

3.6. Soil Organic Carbon

SOC is a part of the global carbon cycle which involves the cycling of carbon through plants and soil. Litterfall in the forest floor is one of the major sources of SOC and this is the main component of SOM [69]. The SOC in the study forest significantly ($p<0.05$) varied with soil depths as well as within the three land uses. It was found that sub-surface soil in the study area contains higher SOC than that of surface soil. At surface soil, the highest mean value of SOC was found in *S. robusta* plantation (1.90±0.19%) and lowest was found in the deforested land (0.32±0.20%). At sub-surface soil, the highest mean value of SOC was found in *D. turbinatus* plantation (3.47±0.10%) followed by *S. robusta* plantation (2.79±0.23%) (Table 4). The average of the mean value of SOC was found in *D. turbinatus* plantation soil (2.5±0.15%) followed by *S. robusta* plantation soil (2.35±0.21%) (Table 4 & Fig. 2).

The recorded mean value of SOC corresponds with the findings of Osman et al [70] in Bangladesh. Similarly, Akhtaruzzaman et al [53] recorded higher SOC in the planted forest soil as compared to barren soil and cultivated land soil can be ascribed to the addition of OC from tree cover. Mia et al [61] also found a higher amount of SOC in a mixed forest stand in comparison to pure forest stand and lowest in the plantation forest stand. In addition to that, some studies e.g., Akhtaruzzaman et al [52-53] and Shaifullah et al [63] estimated that the surface soil contain higher SOC might be attributed to the higher accumulation of SOM on the surface soil.

3.7. Soil Carbon to Nitrogen Ratio (C: N)

Soil carbon to nitrogen ratio (C: N) determining whether the carbon sink in land ecosystems could be sustained over the long-term [71]. Change in the amount of N in the ecosystem is a key parameter regulating long-term terrestrial carbon sequestration [72]. Soil C:N ratio determines the decomposability of SOM, therefore has an important impact on plant N availability. In the forest floor, C: N ratio is generally wide and decreases as decomposition occurs, in other soils the ratio is usually much lower [73]. The survey found that C:N ratio decreased from plantations sites to deforested site as well as from sub-surface soil to the surface soil. It was found that the mean C: N was higher in surface soil in *D. turbinatus* plantation (14.3±0.02 C/N) than the *S. robusta* plantation (11.4±0.02 C/N). On the other hand, at sub-surface soil C: N was higher in *S. robusta* plantation (24.5±0.03 C/N) than in *D. turbinatus* plantation (23.7±0.03 C/N). The average of mean value of C: N was higher in *D. turbinatus* plantation (19±0.02 C/N) than in *S. robusta* plantation (17.95±0.02 C/N). The mean C: N values were significantly different at soil depths and land uses at $p\leq0.05$ (Table 3 & Fig. 2).

Findings of the Mia et al [61] revealed that the soil C: N was found higher in the pure and mixed forest stands and lowest in the plantation forest stands. Biswas et al [55] found a higher amount of soil C: N in the forested sites in comparison to other land use change including shifting cultivation. Comparable, soil C: N was recorded higher in fallow site soil after 3-years of burning prepared for shifting cultivation than soil of other shifting cultivation sites [49].

3.8. One-way ANOVA Analysis of Soil Physicochemical Properties according to Soil Depths

Table 5 shows the summary results of one-way ANOVA analysis of selected soil physicochemical properties according to surface soil and sub-surface soil at $p<0.05$ level of significance. The ANOVA results showed that the value of soil pH was significant ($P=0.000$) at $p<0.05$ ($F=18.800$) in two soil depths which indicated that there is a significant difference in the mean value of soil pH. The ANOVA results of available K showed that the value of K was significant ($P=0.000$) at $p<0.05$ ($F=15.733$) in the two soil depths which also indicated that there is a significant difference in the mean value of available K.

Similarly, ANOVA results of available P value showed that the value was significant ($P=0.000$) at $p<0.05$ ($F=866.705$) among the two soil depths which indicated that there is a significant difference in the mean value of available P. The ANOVA results in total N was significant ($P=0.000$) at $p<0.05$ ($F=46.714$) which showed that there is a difference in the mean value of total N among the two soil depths. Furthermore, the ANOVA test showed that there was a significant difference ($P=0.000$) in

Table 5. One-way ANOVA analysis of soil physicochemical properties according to soil depths and land uses

Soil macronutrients	Soil depth		Land use and land cover	
	F	P	F	P
Soil p^H	18.800	0.000	12.885	0.001
Available K	15.733	0.000	2.803	0.092
Available P	866.705	0.000	1.462	0.263
Total N	46.714	0.000	20.960	0.000
SOM	2891.729	0.000	63.744	0.000

the mean value of SOM at $p<0.05$ (F=2891.729) according to two soil depths (Table 5).

3.9. One-way ANOVA Analysis of Soil Physicochemical Properties according to Land Use Change

Table 5 also showed the summary results of one-way ANOVA analysis of selected soil physicochemical properties according to three land uses at $p<0.05$ level of significance. The ANOVA results of soil pH showed that the value of soil pH was significant (P=0.000) at $p<0.05$ (F=12.885) in three land uses which indicated that there is a significant difference in the mean value of soil pH. The ANOVA results of available K showed that the value was significant (P=0.092) at $p<0.05$ (F=2.803) in three land uses which also indicated that there is a significant difference in the mean value of available K.

The ANOVA results of available of P showed that the value was insignificant (P=0.263) at $p<0.05$ (F=1.462) among the two land uses which indicated that there is no significant difference in the mean value of available for P. Besides, the ANOVA results of total N was significant (P=0.000) at $p<0.05$ (F=20.960) which showed that there is a difference in the mean value of total N among the three land uses. Similarly, the ANOVA results showed that there was a significant difference (P=0.000) in the mean value of SOM at $p<0.05$ (F=63.744) according to three land uses (Table 5).

4. CONCLUSIONS

The present study suggested that there was considerable variation among soil physicochemical in two plantation sites and a deforested site, and soil samples from two soil depths within three land uses. The study results showed that the soils under *S. robusta* and *D. turbinatus* plantation forests had higher physicochemical contents in all the cases than deforested site. Soil pH, available K and P value were slightly higher in two plantation sites compared to deforested site. The total N, SOM, SOC and soil C: N value also found higher in two plantation sites than the deforested site. These mean the continuous declining in soil quality which would have a negative effect on the structure, function and productivity of forest ecosystem. Results revealed that the physicochemical properties in the studied eco-park was poor due to less tree coverage, less water-holding capacity and excessive soil erosion during monsoon, however, massive site-specific tree plantation with soil management practices can improve the soil physicochemical properties. Sustainable land use practices like tree plantation with native tree species (timber, fruit-bearing and medicinal plants), agroforestry and regular soil protection by cover crops, inter-cropping and mulches can restore soil fertility and productivity by increasing SOM and water holding capacity in the deforested site. Further research is needed to find out the impact of land use change variations on soil biological properties as well as ways to improve soil quality and ecosystem productivity through more detailed and expanded similar studies.

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Aims and Scope: *Proceedings of the Pakistan Academy of Sciences* is official journal of the Academy, published quarterly, in English. This open access journal publishes research papers in *Engineering Sciences & Technology, Life Sciences, Medical Sciences, and Physical Sciences*. State-of-the-art reviews (~20 pages, supported by recent references) summarizing R&D in a particular area of science, especially in the context of Pakistan, and suggesting further R&D are also considered. Manuscripts undergo double-blind review. Authors are not required to be Fellows or Members of the *Pakistan Academy of Sciences* or citizens of Pakistan.

Manuscript Format

Manuscript may contain **Abstract, Keywords, INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION (or RESULTS AND DISCUSSION), CONCLUSIONS, ACKNOWLEDGEMENTS and REFERENCES** *and any other information that the author(s) may consider necessary.* The Manuscript sections must be numbered, i.e., **1. INTRODUCTION, 2. MATERIALS AND METHODS,** and so on.

Manuscripts, in *Times New Roman*, 1.5-spaced (but single-space the Tables), with line numbering and one-inch margins on all sides on A-4 size paper, should not exceed 20 pages including Tables and Figures. Number manuscript pages throughout. The text (in **Font Size 11,** except for the sections mentioned in **Font Size 10)** must be typed in a single column across the paper width. All Tables and Figures must be placed after the text, i.e., after REFERENCES section.

(a) **Title** of the article **(**Capitalize initial letter of each main word; font size 16; **bold**), max 160 characters (no abbreviations or acronyms), depicting article's contents; (b) Author' first name, middle initial and last name (font size 12, **bold**), and professional affiliation (i.e., each author's Department, Institution, Mailing address and Email; but no position titles) (font size 12); (c) Indicate the corresponding author with *; (d) **Short running title**, max 50 characters (font size 10).The **next Page** should start with **Title** of the Article, followed by entire manuscript. **Headings and Subheadings** (font size 11): All flush left

LEVEL-1: **ALL CAPITAL LETTERS; bold**

Level-2: **Capitalize each main word; bold**

Level-3: Capitalize each main word; Bold, Italic

Level-4: *Run-in head*; *Italics*, *in the normal paragraph position. Capitalize the initial word only and end in a colon (i.e., :)*

Abstract (font size 10; max 250 words)**:** Must be self-explanatory**,** stating rationale, objective(s), methodology, main results and conclusions of the study. Abbreviations, if used, must be defined on first mention in the Abstract as well as in the main text. Abstract of review articles may have variable format.

Keywords (font size 10)**:** Three to eight keywords**,** depicting the article.

INTRODUCTION: Provide a clear and concise statement of the problem, citing relevant recent literature, and objectives of the investigation.

MATERIALS AND METHODS: Provide an adequate account of the procedures or experimental details, including statistical tests (if any), in a concise manner but sufficient enough to replicate the study.

RESULTS: Be clear and concise with the help of appropriate Tables, Figures and other illustrations. Data should not be repeated in Tables and Figures, but must be supported with statistics.

DISCUSSION: Provide interpretation of the RESULTS in the light of previous relevant studies, citing published references.

ACKNOWLEDGEMENTS (font size 10)**:** In a brief statement, acknowledge financial support and other assistance.

REFERENCES (font size 10)**:** Cite references in the text **by number only** in **square brackets**, e.g. "Brown et al [2] reported ..." or "... as previously described [3, 6–8]", and list them in REFERENCES section, in the order of citation in the text, Tables and Figures (not alphabetically). Only published (and accepted for publication) journal articles, books, and book chapters qualify for REFERENCES.

List of REFERENCES must be prepared as under:

a. **Journal Articles** *(Name of journals must be stated in full)*

1. Golding, I. Real time kinetics of gene activity in individual bacteria. *Cell* 123**:** 1025–1036 (2005).
2. Bialek, W. & S. Setayeshgar. Cooperative sensitivity and noise in biochemical signaling. *Physical Review Letters* 100**:** 258–263 (2008).
3. Kay, R.R. & C.R.L. Thompson. Forming patterns in development without morphogen gradients: differentiation and sorting. *Cold Spring Harbor Perspectives in Biology* 1: doi: 10.1101/cshperspect.a001503 (2009).

b. **Books**

4. Luellen, W.R. *Fine-Tuning Your Writing*. Wise Owl Publishing Company, Madison, WI, USA (2001).
5. Alon, U. & D.N. Wegner (Ed.). *An Introduction to Systems Biology: Design Principles of Biological Circuits*. Chapman & Hall/CRC, Boca Raton, FL, USA (2006).

c. **Book Chapters**

6. Sarnthein, M.S. & J.D. Stanford. Basal sauropodomorpha: historical and recent phylogenetic developments. In: *The Northern North Atlantic: A Changing Environment.* Schafer, P.R. & W. Schluter (Ed.), Springer, Berlin, Germany, p. 365–410 (2000).
7. Smolen, J.E. & L.A. Boxer. Functions of Europhiles. In: *Hematology, 4th ed*. Williams, W.J., E. Butler & M.A. Litchman (Ed.), McGraw Hill, New York, USA, p. 103–101 (1991).

Tables, with concise but self-explanatory headings must be numbered according to the order of citation (like **Table 1**, **Table 2**). Round off data to the nearest three significant digits. Provide essential explanatory footnotes, with superscript letters or symbols keyed to the data. Do not use vertical or horizontal lines, except for separating column heads from the data and at end of the Table.

Figures may be printed in two sizes: column width of 8.0 cm or page width of 16.5 cm; number them as **Fig. 1**, **Fig. 2**, … in the order of citation in the text. Captions to Figures must be concise but self-explanatory. Laser printed line drawings are acceptable. Do not use lettering smaller than 9 points or unnecessarily large. Photographs must be of high quality. A scale bar should be provided on all photomicrographs.

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Reviewers: Authors may suggest four relevant reviewers, two local and two from scientifically advanced countries.

Manuscripts must be submitted in Microsoft Word (.doc or .docx format; **pdf** files not acceptable). Figures can be submitted in Word format, TIFF, GIF, JPEG, EPS, PPT.

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