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Dear Esteemed Readers, Authors, and Colleagues,

I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

At Integrative Omics and Applied Biotechnology (IIOAB) Journal, we firmly believe in the transformative power of science and innovation, and we recognize that it is the vigor and enthusiasm of young minds that often drive the most groundbreaking discoveries. We actively encourage students, early-career researchers, and scientists to submit their work and engage in meaningful discourse within the pages of our journal. We take pride in providing a platform for these emerging researchers to share their novel ideas and findings with the broader scientific community.

In today's rapidly evolving scientific landscape, it is increasingly evident that the challenges we face require a collaborative and interdisciplinary approach. The most complex problems demand a diverse set of perspectives and expertise. Integrative Omics and Applied Biotechnology (IIOAB) Journal has consistently promoted and celebrated this multidisciplinary ethos. We believe that by crossing traditional disciplinary boundaries, we can unlock new avenues for discovery, innovation, and progress. This philosophy has been at the heart of our journal's mission, and we remain dedicated to publishing research that exemplifies the power of interdisciplinary collaboration.

Our journal continues to serve as a hub for knowledge exchange, providing a platform for researchers from various fields to come together and share their insights, experiences, and research outcomes. The collaborative spirit within our community is truly inspiring, and I am immensely proud of the role that IIOAB journal plays in fostering such partnerships.

As we move forward, I encourage each and every one of you to continue supporting our mission. Whether you are a seasoned researcher, a young scientist embarking on your career, or a reader with a thirst for knowledge, your involvement in our journal is invaluable. By working together and embracing interdisciplinary perspectives, we can address the most pressing challenges facing humanity, from climate change and public health to technological advancements and social issues.

I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.

Yours sincerely,

Vasco Azevedo

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RESEARCH ARTICLE OPEN ACCESS

ROLE OF MACROPHYTES IN A SEWAGE FED URBAN LAKE

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ABSTRACT

Macrophytes play a major role in maintaining the nutrient levels in urban aquatic systems. However their prolific growth result in spread of invasive species such as water hyacinth (Eichhornia crassipes) due to the availability of higher nutrient concentrations. This hinders aerobic functioning of the lake by restricting sunlight penetration and also affecting algal photosynthesis. This also results in anoxic environment due to blockage of air-water interface, influencing oxygen diffusivity. Reduction in DO (0 mg/l) impacts the viability of aquatic biota and result in the disappearance of biodiversity. This communication evaluates the influence of the invasive macrophytes in the functioning of lake across the seasons. Significant seasonal changes in water quality were noticed due to changes in the redox conditions (- 235 mV) and dissolved oxygen levels at various locations depending on the extent and location of macrophyte spread based on the nutrient levels coupled with wind regime prevailing during the season. The analysis of seasonal data reveals that dissolved oxygen concentration and redox condition is dependent on the extent of macrophyte spread. N content in Lemna and Alternanthera species (of 4 g/100 g dry weight) is significant compared to other species ($p<0.005$). During monsoon, lake functions in the absence of macrophytes, predominantly as aerobic lagoon; and functions as aerobic-anaerobic lagoon (pre-monsoon) and as anaerobic–aerobic system (post-monsoon). Anaerobic conditions are mainly due to the interference of macrophytes in lake functioning and inefficient handling of nutrients in the absence of algae. This necessitates the regular removal of macrophytes from the lake. Provision to allow the growth of primary producers will help in nutrient management.

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KEY WORDS

macrophytes; wastewater; nutrients; eutrophication; lagoon; sewage; urban lakes

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[I] INTRODUCTION

Macrophytes grow in or near water and are emergent, submergent, or floating, forming a vital component of lake ecosystems. However, the introduction of invasive-exotic species such as water hyacinth (*Eichhornia crassipes*), alligator weed (*Alternanthera phyloxiroides*), water lettuce (*Pistia stratiotes*) etc. have changed the lake dynamics significantly. In recent times the urban waterbodies are being used for the disposal of sewage, etc. Sustained inflow beyond the assimilative capacity of waterbodies has lead to eutrophication, resulting in the profuse growth and spread of invasive species. Influx of partially treated and untreated sewage has resulted in overgrowth, ageing, and subsequent decay of macrophytes creating anoxic conditions and devouring the system from life giving oxygen. This has impacted the food chain and hence the ecological integrity of the system.

Water hyacinth (*Eichhornia crassipes*) native to Brazil has been introduced to tropical and subtropical region, [1] is amongst the fastest growing, free floating freshwater invasive weed species which derives required nutrients directly from water. Its distribution and dispersal is aided by water currents and wind. It consists of 5% dry matter with 50% silica, 30% potassium, 15% nitrogen and 5%protein[2]. Its potential negative characteristics pose a threat for the habitat quality of waterbodies. The average growth rate of water hyacinth is 10-12 g/m^2/d and the maximum is 45-50 g/m^2/d [3, 4, 5]. During growth, water hyacinth can store N up to 909 g/m^2 [6]. These invasive aquatic plants form a thick 'mat' that restricts the exchange of oxygen across the air/water interface and also hampers algal photosynthesis resulting in reduced dissolved oxygen. The anoxic conditions under water hyacinth mats also favour the release of nitrogen and phosphorous (N and P) from sediments which may further aid the rapid growth of macrophytes [7, 8, 9]. In addition, it influences the wind-driven water movement, impeding circulation of oxygen-rich surface water [10]. Bank side grasses grow over the water hyacinth mats, anchoring the mats to the bank edges. Varieties of grasses and sedges as *Cyperus* sp. and in some instances, plants like *Colocasia esculenta* (taro) etc. have established themselves on these mats. Once established, very large flows are required to break them up and disperse.

The southwest monsoon winds tend to push the floating macrophytes over spillways of lakes situated on their south-eastern, eastern and southwestern edges, thereby ridding the water surface free of macrophytes each year. This natural flushing of macrophytes during monsoon associated with the

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phenological events was considered to be the most important short-term process for cleaning urban lakes. The macrophytes in their matured stage are infested by the mottled water hyacinth weevil and caterpillar that reduces about 75% of the leaf surface areas in 2-3 weeks, consequently resulting in loss of the major photosynthesizing machinery i.e. the leaves and greatly helps in compacting the water hyacinth mass, as they also disrupt the long, spongy and bulbous stalk tissues, the plants lose their buoyancy and settles faster which is followed by leaching of plant nutrients and subsequently rapid bacterial degradation takes place which reduces the DO levels significantly and creates anaerobic conditions throughout the lake. Thus this process submerges a large quantity of organic matter which ultimately decomposes, increasing the biochemical oxygen demand (BOD) that deteriorates water quality. Dissolved oxygen falls to such low level that leads to massive fish kills [11].

Oxygen is amongst the most important of several dissolved gases vital to aquatic life. It is a principal and direct indicator of water quality in surface waters. Primary source of oxygen in surface water is from photosynthesis of aquatic plants, algae and diffusion of atmospheric oxygen across the air water interface. The dissolved oxygen content of natural water varies with the temperature, photosynthetic activities and respiration or decomposition of plants and animals [12]. On a daily basis they maintain equilibrium as per the consumption and production. The diurnal oxygen cycle varies in a sinusoidal manner with minimum values observed early in the morning and maximum concentrations at midday [13]. A decline in DO has serious implications on the health of the aquatic system, as hypoxic and anoxic conditions reduce or eliminate sensitive native fish and invertebrate species.

During aerobic decomposition, cellulosic materials are converted into carbon dioxide and water by the bacterial action. CO_2 in the dissolved form maintains equilibrium with its carbonate and bicarbonate forms and decides the C supply for the algae and aids in photosynthesis bringing manifold increase in the primary productivity of the system. Oxygen level of the waterbodies are reduced by continuous inflow of sewage, containing large loads of organic carbon, phosphates and nitrates that finally lead to profuse growth and spread of aquatic biota. Under such circumstances, aquatic plants and algae proliferate incredibly and when they die they form food for bacteria, which in turn multiply and use large quantities of dissolved oxygen. In addition to this, when plant biomass increases at the surface of the water (pelagic zone) they block transmittance of sunlight into deeper layers and diffusion of oxygen from the atmosphere into the water, thereby, reducing photosynthetic potential of submerged plants and algal species. In addition to this, their extensive root system in the water provides a large surface area for the growth of microbes which rapidly consume DO [14]. These microbes render the system more anoxic by carrying out the anaerobic digestion on a myriad of substrates. Moreover, under anoxic conditions, ammonia, iron, manganese and hydrogen sulphide concentrations can rise to levels deleterious to biota. In addition, phosphate and ammonium are released into the water from anoxic sediments further enriching the ecosystem [15].

Varthur lake, situated in the south of Bangalore, was built to store water for drinking and irrigation purposes [16]. However, over the last five decades, due to sustained influx of sewage, nutrients in the lake are now well over safe limits. Sewage brings in large quantities of C, N and P which are trapped within the system. This lake receives about 40% of the city sewage (c.500million liters per day, MLD) resulting in eutrophication. There have been substantial algal blooms, dissolved oxygen depletion and malodour generation, apart from extensive growth and spread of water hyacinth that covers about 85% of the lake during the dry season.

Water hyacinth mats greatly reduces DO content in water under the mats [17, 8, 9] affecting aquatic diversity and productivity. Decomposition of macrophytes happens due to ageing, over-crowding, wind driven compaction, pest damage, etc. During oxidation, microflora utilize detritus C as an energy source and reduces electron acceptors such as oxygen, nitrate and sulphate [18]. Water hyacinth litter breaks down as a result of aerobic, anaerobic and facultative anaerobic microbial activity [19]. Bacteria accentuate degradation process and fungal decomposition under such conditions is negligible [20]. O_2 concentrations in water play an important role in the release and transformations of nutrients [21].

This paper focuses on the impact of wind induced drift of macrophytes, its removal during monsoon, and its rapid growth which governs the aerobic-anaerobic status of the lake and thereby brings out its relation with the water quality. The objectives of the study were to:

i. Determine the major contributor of the BOD load that disrupts the lake's functioning,
ii. Map oxic, hypoxic and anoxic zones based on DO levels and to understand the influence of wind induced drift of macrophytes on seasonal water quality changes and
iii. Quantify nutrient loads (C and N) and their uptake by macrophytes.

[II] MATERIALS AND METHODS

The field study was conducted in Varthur lake (12°57'24.98" - 12°56'31.24" N, 77°43'03.02" - 77°44'51.1"E) situated in the south of Bangalore, **[Figure-1]** which is the second largest lake in the city. It covers a water-spread area of 220 ha (maximum depth 2 m) and has a varying extent of floating macrophytes during different seasons. It is a part of a series of interconnected and cascading waterbodies. The Varthur lake catchment has seen large scale land use changes after 2000, following rapid urbanization.

Water samples were collected at 10-15 cm from the surface (to avoid floatables and macrophyte debris), every month over a

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period of twelve months and analyzed for various physico-chemical parameters-pH, water and air temperature, conductivity, turbidity, redox potential and dissolved oxygen (DO), BOD, COD and inorganic nutrient as per standard protocol of APHA [22]. The biomass/macrophyte coverage over the lake surface was also monitored with the help of GPS and remote sensing data. For macrophyte biomass estimation, 1 m^2 quadrate sampling method was adopted [23]. C and N contents were determined using CHN analyzer. The algal community structures at various sampling sites were also investigated. The nutrient content in water and biomass were analyzed. The pattern of the wind induced drift resulting in the movement of macrophyte population and the accumulation at different extremes of the lake was studied. Changes in the dissolved oxygen concentration and other water quality parameters were investigated with the macrophyte cover and resultant oxidizing or reducing environment.

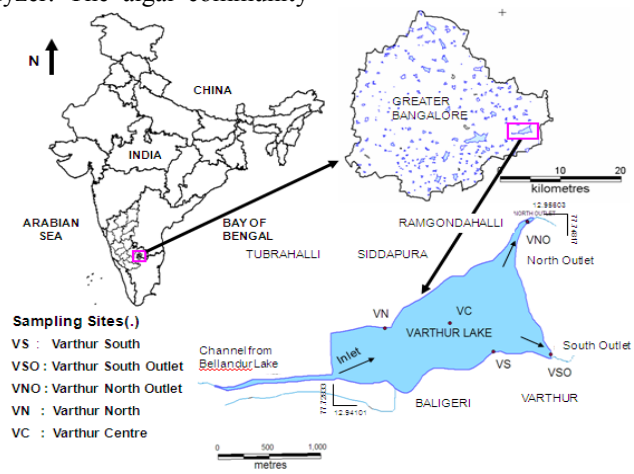

Fig: 1. Varthur lake, Greater Bangalore, India with sampling locations

[III] RESULTS AND DISCUSSION

3.1. Monthly variations of dissolved oxygen (DO) concentrations

Water quality parameters were monitored on monthly basis **[Table-1]**. Significant variations in mean monthly values of DO were observed **[Figure-2]**. DO ranged from 0-5 mg/l depending on the extent and density of macrophytes during the morning. In anthropogenically modified, weed-infested streams from upper reaches, deoxygenated water (DO = 0-0.3 ppm) arrives for most part of the day, due to high flow rates of water through extensive weed mats. The influx of hypoxic, nutrient rich wastewaters during the mid day is more stressful to aquatic biota as fish and invertebrates that undergo higher metabolic rates during the day require a higher DO than in the night.

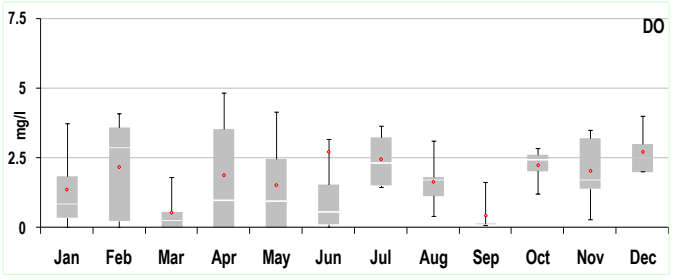

Fig: 2. Month-wise variations in Dissolved Oxygen

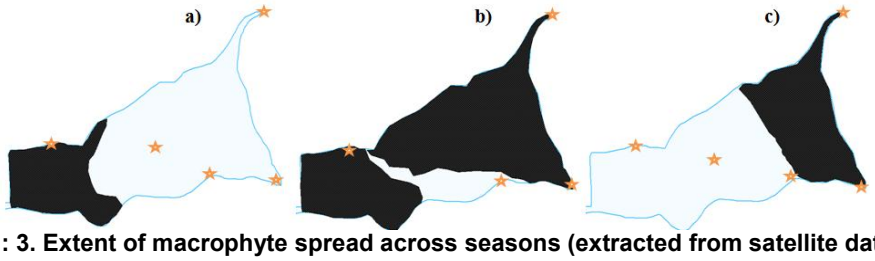

Fig: 3. Extent of macrophyte spread across seasons (extracted from satellite data)
Note: a) Winter b) Summer and c) Monsoon.

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According to BIS (IS 10500-1991) and CPCB standards, the oxygen saturation for surface waters should be around 75% (6 ppm), yet it is the minimum that decides the aptness of habitat for various species (rather than average-based guidelines). Most of the DO data for urban Indian lakes are spot one time measurements during daylight hours, when DO would be substantially above its minimum and in many cases, even approaching its maximum level. Such approaches are inadequate for determining the DO status of these urban waterbodies. Spot readings are used when oxygen levels are typically at their lowest and potentially most stressful for aquatic biota when there is an absence of continuous data [24].

Parameters	Units	μ	σ	Min	Max
Nitrates	ppm	0.304	0.219	0.1	0.96
Ammonium	ppm	15.06	7.6	3.93	30.73
Phosphates	ppm	0.98	0.7	0.14	3.5
Total Phosphates	ppm	7.86	2.44	3.14	9.87
BOD	ppm	89.65	38.54	44	186.1
COD	ppm	98.2	21.24	52	197.3
pH	units	7.61	0.64	6.2	8.22
EC	μS/cm	1054.4	158.64	751	1420
DO	ppm	1.56	0.67	0	13
Transparency	cm	23	3.16	18	28
Turbidity	NTU	78.5	25.6	29	224
ORP	mV	-9.33	129.29	-235	135

Table: 1. Physico-chemical parameters of Varthur lake.
Note: μ: mean; σ: standard deviation; Min: minimum, Max: maximum.

Field investigations reveal that DO is correlated to temperature (r = 0.79). Higher DO levels during the mid-day are due to enhanced algal photosynthetic activities with higher insolation. However during the night due to respiration of aquatic biota, DO levels drop to zero. Furthermore, higher variability in DO was observed during summer. In stagnant systems, which are not light limited, minima is typically around dawn, but in flowing conditions, upstream conditions, flow rates and mass loading make this less predictable.

3.2. Spatial analysis and seasonal effects on wind-induced macrophyte drift and consequent deposition

Varthur is a shallow, wind-influenced hypereutrophic lake characterized by consistent phytoplankton blooms and having higher deposits of unconsolidated organic sediment. The lake receives about 500 MLD sewage (measured) which undergoes anaerobic stage in the upper reaches of the lake. BOD at the inlet is about 120-200 mg/L, Algae driven oxidative BOD reduction facilitate the water to be oxic and brings down the BOD to about 30mg/L at outlets when the lake is not infested with exotic weeds. The algal population plays a pivotal role in maintaining the oxic condition's of the water. Preponderance of ammonia (~40 ppm; **[Table-1]**) at critical levels when the lake is infested with macrophytes poses a threat to the lake's aquatic biological food chain and its activities. The wind regime plays a decisive role in the spread and location of macrophytes mats in the lake. A study conducted to understand the DO levels in various locations of the lakes, reveals significant differences in the dissolved oxygen depending on presence or absence of macrophytes cover. The DO values were monitored in various seasons during the study period to address seasonal variability's. **[Table-2]**.

During the pre-monsoon summer period, the macrophytes grows luxuriously all over the surface of the lake **[Figure-3b]** thus creating anoxic zones (Oxidation reduction potentials ORP -65 to -225 mV), along with enhanced bacterial activities under higher reigning temperatures. Roots of the floating macrophytes provide a good substratum for the attachment of bacteria, drastically reducing the DO levels and resulting in hypoxia and anoxia. DO varies depending on the extent and density of macrophyte mats, evident from the significant difference ($p=0.00006<0.001$) **[Table-3]** in regions with or without macrophytes. This emphasize that lake functioned as anaerobic lagoon. The floating mat of macrophytes gets compacted with an anoxic environment just beneath it. With the increased amount of plant litter decomposition, it significantly contributes to higher autochthonous organic load and hence BOD. The DO values reveal consistent anoxic zones associated with the macrophytes and thus the seasonal changes in the pattern of oxygenation at various extremes of the lake.

During June, gusty westerly winds (4.7 m/s) drifts water hyacinth towards outlets **[Figure-3c]** and subsequent drifts compact water hyacinth which forms thick mat in the region. This compaction is aided by the pest infestation and ageing, which further helps in compacting and also reducing the biomass. This aids in rapid settling while decomposition often creates an anoxic environment near the outlets. The regions near the outlets were highly anaerobic (ORP -180 to -218 mV) with DO values 0 mg/l, compared to the upper reaches which were free from macrophytes (ORP +70 to +85) with DO values from 6.5 to 11.5 mg/l. DO concentrations at outlets were significantly different ($p=1.1 \times 10^{-12} <0.0001$) **[Table-3]** from the regions free of macrophyte cover (inlet and middle regions).

During monsoon, higher catchment run-off into the lake pushes macrophytes including decomposed, semi-decomposed plant litter to the downstream. This exposes water surface to air and sunlight allowing photosynthetic activities in the lake aiding algal growth **[Figure-3a]**. This process rejuvenates the system to aerobic status. Furthermore higher inflow help in cleansing the system from superficial sludge accumulated at outlets which improves the system's performance. The sludge up-welled by wind turbulence comprises of semi-degraded macrophyte biomass (C: N = 50.05:3.02) showing that most of the C forms are intact. However lower values of N indicates uptake by micro organisms, algae and macrophytes.

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Table: 2. DO concentrations at the mid-day at various sites in all seasons

Sampling Location	DO Concentration at Mid-Day (ppm)		
	Pre-monsoon	Monsoon	Post-monsoon
Inlet	8	2.5	3.73
Centre	9.5	6.5	0.13
Outlet North	0	1.3	0.0
Outlet South	0	12.6	0.48
South	12	8.2	1.2

Table: 3. One way ANOVA for DO concentrations in all four studied seasons

Dependent Parameter	Source	Degree of Freedom	F value	p-value at < 0.00001
Summer DO	A	1	23.56	6×10^{-5}
	B	24		
	C	25		
Winter DO	A	2	24.99	4.16×10^{-5}
	B	24		
	C	25		
Monsoon DO	A	1	29.02	6.04×10^{-5}
	B	16		
	C	17		
Spring DO	A	1	396.93	1.1×10^{-12}
	B	16		
	C	17		

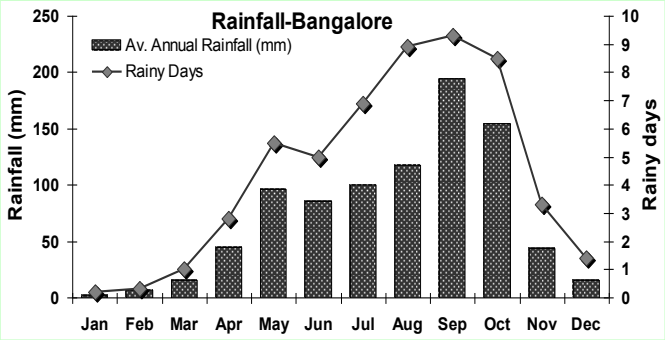

Fig: 4. Monthly rainfall variations near the Study area

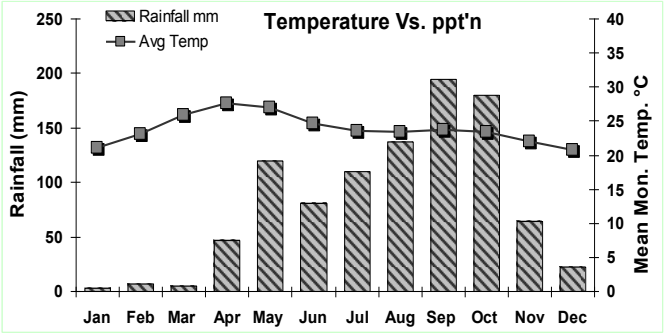

Fig: 5. Comparison between Mean monthly temperatures with the precipitation.

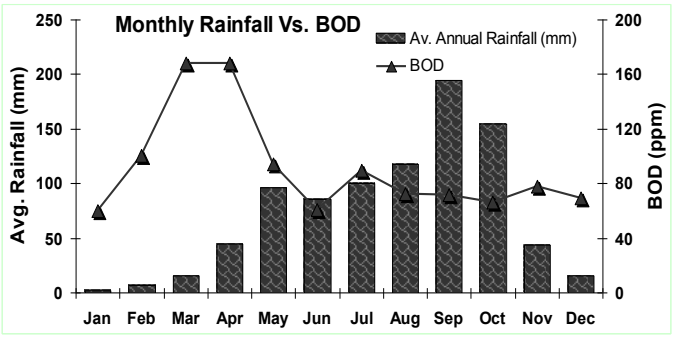

Fig: 6. Relation between the precipitation and mean BOD values

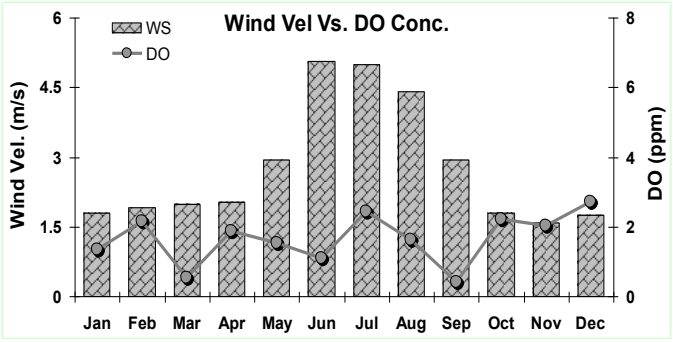

Fig: 7. Relation between the monthly wind velocities with avg. monthly DO level

3.3. Algal seasonal dynamics in the lake and role in supplementing DO level

Algal communities identified upto the genus levels shows algal species from four different families **[Table-4]**. During summer *Scenedesmus* sp., *Anabaena* sp. and *Anaycstis* sp. were dominant while enormous *Chlorella* sp. was observed during monsoon season (80 %). Micro-algal sampling studies also revealed a greater dominance of diatoms especially *Nitszchia* sp. near the inlet reaches during the summer and

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euglenophycean members like *Euglena* sp. and *Trichellomonas* sp. dominated in the monsoon. Filamentous algae's like *Oedogonium* sp. and *Oscillatoria* sp. were observed near outlets. Comparative analysis of algal populations in biofilms showed a marked difference in the community structures in various zones of the lake. Diatom species such as *Gomphonema* sp. and *Nitszchia* sp. at the inlet and chlorophytes and euglenoides were observed at the outlets. Field investigations reveal that there is a periodic transition from an anaerobic-aerobic (in monsoon) to anaerobic (in summer) and aerobic-anaerobic system (winter/pre monsoon) as algae play a vital role in oxygenating the system that lowers BOD. This depends upon the wind direction and the extent of growth and movement of the macrophytes together with the nutrient influx.

Table: 4. Algae communities identified upto genus level

Chlorophyceae	Cyanophyceae	Bacillariophyceae	Euglenophyceae
Chlamydomonas	*Cylindrospermo*	*Gomphonema*	*Phacus*
Chlorogonium	*-psis*	*Cymbella*	*Euglena*
Scenedesmus	*Arthrospira*	*Navicula*	*Trachelomonas*
Ankistrodermus	*Microcystis*	*Pinnularia*	*Lepocinclis*
Chlorella	*Oscillatoria*	*Nitzschia*	
Oedogonium	*Anabaena*	*Synedra*	
	Merismopedia	*Fragillaria*	
	Lyngbya	*Cocconeis*	
		Melosira	

3.4. Characteristic change in water quality and its improvement after flushing out of macrophytes by wind and water flow

The rainfall pattern shows an increase in the intensity mostly during August, September and October **[Figure-4]**. During pre-monsoon period dense mats of water hyacinth and other weeds covering 85% of surface had contributed to low DO levels that are detrimental for the phytoplankton. Faster decomposition of macrophytes and algal organic biomass due to high temperature (summer) **[Figure-5]** resulted in very high BOD values. The degree of mineralization and bacterial respiration was also very high at this time. BOD values were found to be lower during the other seasons especially in monsoon **[Figure-6]**. Prevalence of hypoxic conditions below critical thresholds over a long period is detrimental to the survival of aquatic biota. Onset of monsoon with higher wind velocities and higher catchment run-off allow the water surface to sunlight and re-aeration, enhancing DO levels **[Figure-7]**. The improvement included greater diurnal cycling **[Figure-8]**, both higher maxima and minima and reduced amount of time spent below the adopted 25% threshold for ensuring survival of all naturally occurring biota.

3.5. Macrophyte spread and DO levels

Dense macrophyte mats limit re-aeration by isolating the air/water interface [24] and block sunlight, limiting photosynthetic oxygen production. The concentration of DO in the lakes diminish as a result of biodegradation of carbonaceous and nitrogenous wastes discharged into the waterbodies, deposited in the sediment and the influx of plant limiting nutrients which leads to eutrophication. [25]. In addition, the large organic load created by water hyacinth mats and other vegetation associated with these mats, increase oxygen consumption [26, 27], and they act as a physical substrate for microbes, the metabolic activity of which further increases oxygen demand [26, 24, 9]. Additionally, the extent of water hyacinth infestation within the lagoons may modify edge roughness, water depth and current velocity allowing flowing water to pass through the middle layers of the water column thus reducing the detention time and greatly inhibiting mixing and re-aeration within the lake [26, 28]. In tropical semi-arid zone lakes, there are also substantial variations in DO between different periods of a day where occasional low DO levels can result in the elimination of key aquatic species. In the case of eutrophic lakes though the DO levels become supersaturated at the mid-day, there are chances of DO reaching 0 ppm due to respiration at night when the concentration of algal biomass is very high and bacteria as well as aquatic biota compete for oxygen resulting in anoxia at night.

During summer around 85% of the exposed water surface area is packed with macrophytes. Total N trapped in the biomass accounts to 1.8 ktons (for a macrophyte cover of 85% in a water spread area of 220 hectares) as water hyacinth can store 1 kg/m^2. Significant diurnal (January and April 2009) variations of DO levels in water were observed to be influenced by the macrophytes in the lake **[Figure-8, 9]**. Figure 8 shows DO measured at the south outlet when it is free of water hyacinth, while the **Figure-9** shows lower DO values measured near the macrophyte infested area which represents restriction of algal growth and algae driven photosynthesis. There was no improvement in the DO levels of the north outlet because of persistent stagnation and the presence of floating macrophytes. As the water flow passes the macrophytes, it undergoes an anaerobic phase, thereby bringing down the DO levels to zero. **Figure-9** gives a comparison between the inlet and outlet DO concentrations, during the dense macrophyte cover.

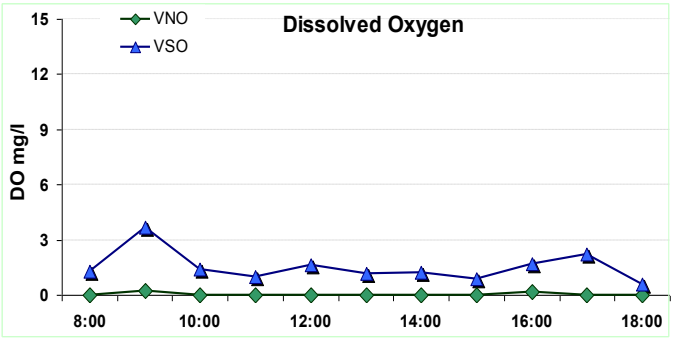

Fig: 8. Diurnal changes of DO levels during April 2009 (summer) at north outlets.

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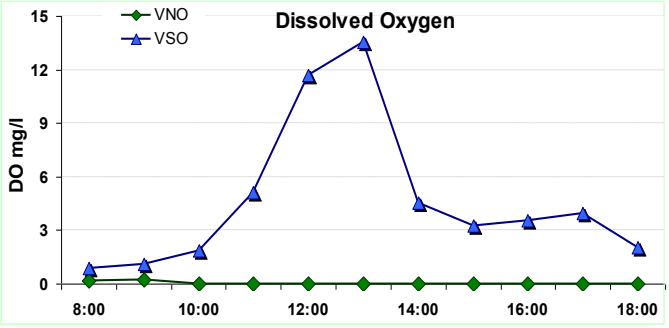

Fig: 9. Diurnal variations in DO concentrations at inlet and outlets

Upstream conditions greatly influences the downstream DO. Improvement in the oxygen content in the Varthur lake outlets shows increasing DO levels with flow. When water is released into storm water drains, its oxygen level is high. However, the oxygen content rapidly diminishes as water move downstream due to mixing of fresh sewage and prevailing anoxic conditions with high organic load and infestation of weeds. This necessitates the clearance of the macrophytes/weeds. Macrophyte removal in the upstream and increasing DO levels at earlier stages would improve the quality of water discharged and that accumulating in the downstream lakes.

3.6. Modified flows, nutrients, proliferation of water hyacinth and rapid nutrient uptake

There is a dynamic interaction between flow, habitat condition and DO saturation in these lakes. Almost all lakes in Bangalore region receive a continuous supplemental dry season flow from sewage or the adjacent agricultural fields. Although the nutrient concentration in the water of Varthur lake was more or less similar with respect to nitrates but an increase in phosphate concentration was observed in summer which correlates positively with the growth of macrophytes and conducive environments for the release of nutrients trapped in the sediments.

The bulk of nutrient uptake during the summer season is performed by the widespread free floating macrophytes. These macrophytes which mainly comprises of water hyacinth and *Alternanthera sp*. covering a substantial portion of the lake surface (85%) captures about 4.5 tons of N/day as depicted in the earlier figure. They propagate very fast with a very high growth rate and engulf the entire water surface in about three months.

The nutrient (C and N) content of the dominant macrophyte population in the lake (from left to right) was investigated. In the lake 10 macrophyte species were observed out of which five dominant macrophyte species arranged as per their abundance from left to right are plotted against their % N content **[Figure-10]**. Higher N content were observed in case of *Lemna gibba* and *Alternanathera phyloxiroides* ~4 g/100 g of dry wt., followed by water hyacinth (2.3 g/100 d of dry wt), *Typha augustifolia* (1.5 g/100 d of dry wt) and *Cyperus* sp. (1.2 g/100 d of dry wt). In other studies, the highest N content was found in *Potamogeton trichoides* Cham. (2.33 g/100 g dry wt.) and *Baldellia ranunculoides* (L.) Parl (2.26 g/100 g dry wt.)[29]. The study conducted in an agricultural drainage lake showed an N content of 2.65 g N /100 g dry wt. in *Potamogeton nodosus* Poir [30]. The N content in *Lemna gibba* in treating the domestic primary effluent in Israel was recorded to be 4.3 % dry wt. which is comparable with the present studies [31]. The study on growth and nutrient storage of water hyacinth showed that 1.6 g N/100 g of dry wt was stored under condition of higher productivity [32].

The study shows that *Alternanthera* sp. together with water hyacinth would have been a dominant accumulator of nutrient in NH_4-N forms. However there was no significant variation in the C content **[Figure-11]** among the major macrophyte species.

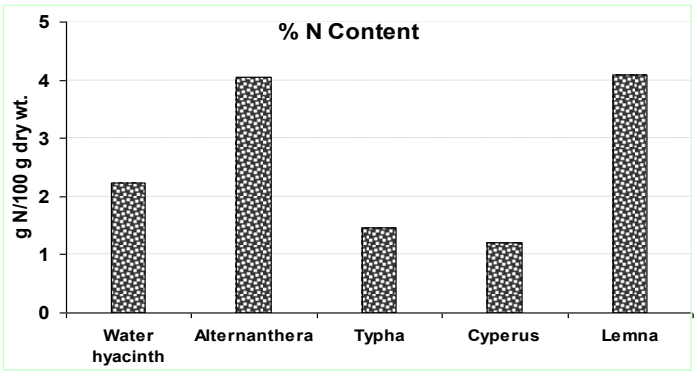

Fig: 10. Variations in percent N content among the dominant macrophytes from left to right.

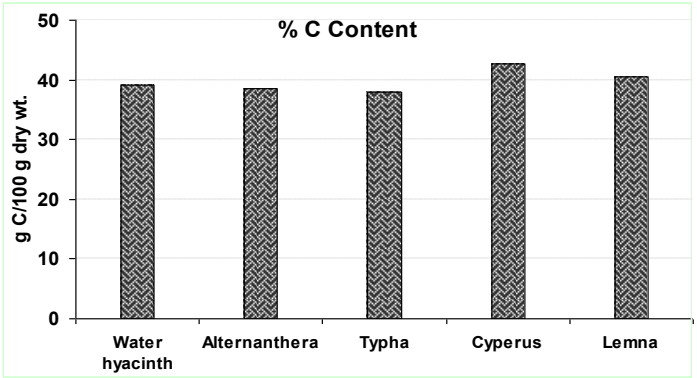

Fig: 11. Variations in percent C Content among the dominant macrophyte species.

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3.7. Plans for the management of macrophytes and associated lower levels of DO

Management arrangements need to recognize that when macrophyte infestations cover the surface of the lagoons, the quality of the aquatic habitat provided is very poor, and opportunities to provide healthy aquatic habitat in a region where most of the existing urban wetlands have been lost or seriously degraded, should be utilized. It is suggested that harvesting and removal of aquatic weeds from the lake rather than letting them sink to the bottom is a necessary prophylactic need. However, due to the large water hyacinth biomass and associated weeds, it was felt that their decomposition process (involving bacteria) would have resulted in significant consumption of the limited DO. Unmanaged exotic aquatic weeds consistently results in poor water quality and reduces the economic value of these otherwise productive habitats. Although the lake examined in this study is impacted by many factors such as altered hydrological regime, increased turbidity and nutrient loads, loss of their riparian zone and run-off from surrounding agricultural areas, the wind induced compaction and removal of macrophytes showed an immediate and substantial improvement in DO levels which were previously excluded because of the low DO content created by weed infestations.

Given the importance of these urban lakes in terms of their role in the livelihood of poor farmers, hydrological cycling, maintenance of micro-climate, as a sink to enormous pollutants and of their high recreational and commercial values there is an immediate need for a rapid improvement of the health of the system which would benefit to maintain the aquatic ecological integrity with optimal balance in urban aquatic systems. The lakes would be very essential further down the years looking at the serious crisis of water, and needs to be well managed for its sustainable functioning and reuse.

Findings of the study show waterbodies further being degraded by the spread and cover of the aquatic weeds/macrophytes and presses on the issues related to complete breakdown of the urban aquatic systems. The results of this study paves a way for initiation and implementation of aquatic weed control programs under existing Urban infrastructure planning and management.

[IV] CONCLUSION

Macrophyte population in the lake maintains the nutrient levels in urban aquatic systems. The increase in nutrient content (32 t N/d) has resulted in a prolific growth of invasive species. During summer, maximum quantity of nutrients in dissolved form is taken up by the macrophytes that cover almost 85% of the lake surface thereby reducing the nutrient content significantly. The lack of air-water interface hampers the aerobic functioning of the lake. Highly anaerobic conditions (-235 mV) are formed which consequently reduces the DO level further creating anoxia. This invasive macrophyte growth in summer raises the quantity of BOD load to about 180 mg/l on the lake significantly. Severe reduced conditions during summer aids in rapid fall of DO levels as low as 0 mg/l. During monsoon in the absence of macrophytes, lake functions as aerobic lagoon driven by micro-algae with satisfactory nutrient uptake and treatability. However in the pre monsoon the system behaves as an aerobic-anaerobic lagoon and finally in the post monsoon period it behaves as an anaerobic–aerobic system.

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REFERENCES

[1] Gopal B. [1987] Water Hyacinth. *Elsevier Sci. Publ, BV. Amsterdam*. 471.
[2] Makhanu KS. [1997] Impact of Water hyacinth in Lake Victoria..Water and Sanitation for all: Partnerships and Innovations. 23rd Water Engineering and Development Centre Conference Durban, South Africa.
[3] Reddy KR, Sutton DL, Bowes G. [1983] Fresh water aquatic plant biomass production in Florida. *Proc. Soil & Crop Sci. Soc. Fla*. 42: 28–40.
[4] Reddy KR, WF Debusk, [1985] Growth characteristics of aquatic macrophytes cultured in nutrient enriched water: I, water hyacinth, water lettuce and pennywort. *Econ. Bot*.38: 229–239.
[5] Sharma A. [1990] Studies on impact of human activities on the trophic state of a lake at Jabalpur. Ph.D. thesis, R. D. University, Jabalpur, 186 pp.
[6] Reddy KR, Debusk WF. [1987] Nutrient storage capabilities of aquatic and wetland plants. In K. R. Reddy & W. H. Smith (eds), Aquatic plants for water treatment and resource recovery .Mangolia Pub. Inc., Florida: 337–357.
[7] Gutierrez EL, Ruiz EG, Uribe EG, Martinez JM. [2001] Biomass and productivity of water hyacinth and their application in control programs. In: Julien, M.H, Hill MP., Center TD, Jianquing, D. (Eds.), Biological and Integrated Control of Water Hyacinth: Eichhornia crassipes. Proceedings of the Second Meeting of the Global Working Group for the Biological and Integrated Control of Water Hyacinth, Beijing, China, 9–12 October 2000, Australian Centre for International Agricultural Research, Canberra, pp. 109–120.
[8] Masifwa WF, Twongo T, Denny P. [2001] The impact of water hyacinth, Eichhornia crassipes (Mart) Solms of the abundance and diversity of aquatic macroinvertebrates along of the shores northern Lake Victoria, Uganda. *Hydrobiologia* 452:79–88.
[9] Scheffer M, Szaba S, Grangnani A, van Nes EH, Rinald S, Kautsky N, Norberg J, Roijackers RMM, Franken RJM. [2003] Floating plant dominance as a stable state. *Proceedings of the National Academy of Sciences USA* 100:4040–4045.

[10] Smith-Rogers S. [1999] Effect of aquatic weeds on water quality.
[11] Benjamin R, Chakrapani BK, Kar D, Nagarathna AV, Ramachandra TV. [1996] Fish Mortality in Bangalore Lakes, India. *Electronic Green Journal*, Issue-6.
[12] Cunningham WP, Siago BW. [1995] Environmental Science .A global concern, 3rd edition, *WHC Brown publisher*.
[13] Mahapatra DM, Chanakya HN, Ramachandra TV. [2010] Assessment of Treatment capabilities of Varthur Lake, Bangalore, India' Int. *J of Envieonment Technology and Management*, 14(1/2/3/4): 84-102.
[14] Robert GW. [2001] Limnology river and lake ecosystem .3rd edition Academic press. A Harcourt Science and Technology company.9
[15] Hutchinson G E. A Treatise on Limnology. Vol I, Part 1. John Wiley & Sons.
[16] Government of Karnataka. [1990] Karnataka State Gazetteer. Lotus Printers, Bangalore. pp. 970, 16: 215.
[17] WilloughbyN, Watson IG, Lauer S, Grant IF. [1993] An Investigation into the Effect of Water Hyacinth on the Biodiversity and Abundance of Fish and Invertebrates in Lake Victoria, Uganda. NRI Project Report 10066 Ao32g, Natural Resources Institute, Chathan UK.
[18] Fenchel TM, Jorgensen BB. [1977] Detritus food chains of aquatic ecosystems: The role of bacteria. *Adv. Microbial Ecol*. 1: l-58.
[19] Reddy K R, DeBusk W F. [1991] Decomposition of water hyacinth detritus in eutrophic lake water. Hydrobiologia 211: 101-109.
[20] Gaur S, Singhal PK, Hasija S K. [1992] Relative contributions of bacteria and fungi to water hyacinth decomposition. *Aquatic Botany* 43:1–15.
[21] Reddy KR, Sacco PD. [1981] Decomposition of water hyacinth in agricultural drainage water. *J envir. Qual*.10: 228–234.
[22] APHA (American Public Health Association) AWWA WEF 1995. Standard Methods for Examination of Water and Wastewater, 19th edition. Washington DC.
[23] Feldmann T, Nogesh P. [2007] Factors controlling macrophyte distribution in large shallow Lake Võrtsjärv, *Aquatic Botany* 87(1):15–21.
[24] Pearson RG, Butler B, Crossland M. [2003] Effects of cane-field drainage on the ecology of tropical waterways. Report no. 03/04. Australian Centre for Tropical Freshwater Research, James Cook University, Townsville.
[25] Gary LJ, Joseph DB. [1998] The basics of pond aeration. Agriculture and natural resource.
[26] Kaenel BR, Buehrer H, Uehlinger U. [2000] Effects of aquatic plant management on stream metabolism and oxygen balance in streams. *Freshwater Biology* 45: 85–95.
[27] Battle JM, Mihuc TB. [2000] Decomposition dynamics of aquatic macrophytes in the lower Atchafalaya, a large floodplain river. *Hydrobiologia* 418:123–136.
[28] Perna C. [2004] Impacts of agriculture and restoration of the habitat values, Water Quality and Fish Assemblages of a Tropical Floodplain. *Masters of Science Thesis*, James Cook University, Townsville, Australia, p. 144.
[29] Margarita Fern´andez-Al´aez, Camino Fern´andez-Al´aez Eloy B´ecares. [1999] Nutrient content in macrophytes in Spanish shallow lakes. *Hydrobiologia* 408/409: 317–326.
[30] Brian H Hill. [1979] Uptake and release of nutrients by aquatic macrophytes. *Aquatic Botany* 7: 87–93.
[31] Noemi Ran, Moshe Agami, Gideon Oron. [2004] A pilot study of constructed wetlands using duckweed (Lemna gibba L.) for treatment of domestic primary effluent in Israel. *Water Research* 38(9): 2241–2248.
[32] Reddy KR, Agami M, Tucker JC [1989] Influence of nitrogen supply rates on growth and nutrient storage by water hyacinth (Eichhornia crassipes) plants. *Aquatic Botany* 36(1): 33–43.

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REVIEW ARTICLE OPEN ACCESS

ENGINEERING OF MICROBIAL PROTEASES: IMPROVING STABILITY AND CATALYTIC PERFORMANCES

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ABSTRACT

Proteolytic enzymes are becoming more ubiquitous as bacteria and fungi. Some proteases from microbial sources are industrially important enzymes but often have to be improved for their catalytic efficiency and stabilities in solvents and temperature at industrial scales in order to suit their applications. Research on protein engineering of microbial proteases to improve their stability and catalytic performances have been extensively conducted by various researchers across the globe using different molecular approaches vis a vis site-directed mutagenesis (SDM) and directed evolutions (DE). SDM has been extremely useful in substitution of important amino acids of microbial proteases; though its major obstacle is that it is imperative to know the three dimensional (3D) structure of the protease in question. Directed evolutions (DE) subsequently emerged as an alternative to SDM, since the knowledge of the enzymes 3D structure is less significant, though its major drawback has been the creation of large mutant libraries and high through put screening of mutant with desired properties. To overcome the drawback of DE, a flow cytometry based screening system have been recently developed which may likely pave way for efficient and fastest way of screening of mutants with improved desired properties.. Sometimes these two approaches can be applied concurrently to obtain enzymes with novel properties. This review aimed at gathering the disperse literature on the approaches where bacteria and fungi have been chosen as sources of microbial proteases. A recent flow cytometry based screening system for DE of proteases has also been reported.

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[I] INTRODUCTION

Many industrial applications of proteases require enzymes with properties that are non physiological. Protein engineering pave ways for the introduction of predesigned changes into the gene for the synthesis of a protein with an altered function that is desired for designated industrial application. Advances seen in recombinant DNA technology and the ability to selectively exchange amino acids by site-directed mutagenesis (SDM) have been responsible for the alarming progress of protein engineering. Identification of the gene and in-depth knowledge of the three-dimensional structure of the protein in question are the two main prerequisites for rational design aspect of protein engineering. The X-ray crystallographic structures of several proteases have been determined [1, 2, 3]. However, because not all proteins and / or enzymes that the scientist have the knowledge of their gene and three-dimensional structures, directed evolution (DE) emerged as a new promising aspect of protein engineering, where the fundamental knowledge of the gene and the 3D structure is less important. Proteases from bacteria and fungi have been engineered to improve their stability and/or catalytic properties to suit their particular applications.

[II] SITE DIRECTED MUTAGENESIS

This type of mutagenesis is also known as oligonucleotide directed mutagenesis or site-specific mutagenesis, is a molecular biology technique in which a mutation is created at a define site in a DNA molecule. The peculiar feature of this mutagenesis requires that the wild type gene be known. Bacteria and fungi are chosen for this review.

2.1. Bacteria

Subtilisin has been selected as a model system for protein engineering since a lot of fundamental information about this commercially important enzyme is available. Its pH dependence [4] catalytic activity [5, 6]stability to heat or denaturing agents [7, 8], and substrate specificity [9, 10, 11, 12, 13] has been altered through SDM. A slightly reduced rate

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of thermal inactivation was observed for a subtilisin BPN9 variant containing two cysteine residues (Cys22, Cys87) [14, 15]. Oxidation of Met222 adjacent to the Ser221 in the active site of subtilisin reduces the catalytic activity of subtilisin.

The effect of substitution of Met222 with different amino acids revealed that small side chains yield the highest activity. The mutant enzymes Ser222, Ala222, and Leu222 were active and stable to peroxide for 1 h. Probing of the specificity of the S1binding site of Met222 Cys/Ser mutants of subtilisin from *Bacillus lentus* with boronic acid inhibitors revealed similar binding trends for the mutant and the parent [16]. The disulfide bonds introduced into subtilisin away from its catalytic center were shown to possess increased autolytic stability [17]. Higher thermostability of subtilisin E as a result of introduction of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease has been reported [18]. Strausberg et al. has created the environment for stabilization of subtilisin by deleting the calcium-binding loop from the protein [19]. Analysis of the structure and stability of the prototype with the loop deleted followed by SDM resulted in a mutant with native proteolytic activity and 1,000-fold-greater stability under strongly chelating conditions. SDM-mediated substitution of Asn241 buried in the neutral protease of *B. stearothermophilus* by leucine resulted in an increase in thermostability of 0.7 ± 0.1°C [20]. The thermostability of the neutral protease from *Bacillus subtilis* was increased by 0.3 and 1.0°C by replacing Lys with Ser at positions 249 and 290, respectively, whereas the Asp249 and Asp290 mutants exhibited an increased stabilization by 0.6 and 1.2°C, respectively [21].

A protein engineering study was undertaken by Bruinenberg *et al*,[22] to determine the functions of one of the largest loop insertions (residues 205 to 219), predicted to be spatially close to the substrate-binding region of the SK11 protease from *L. delbrueckii* and susceptible to autoproteolysis [22]. Deletion or modification of this loop was shown to affect the activity and autoprocessing of the protease. Graham *et al,* [23] showed that random mutagenesis of the substrate-binding site of a-lytic protease, a serine protease secreted by the soil bacterium *Lysobacter enzymogenes*, generated enzymes with increased activities and altered primary specifities Substitution of His120 by Ala in the LasA protease of *Pseudomonas aeruginosa* yielded an enzyme devoid of staphylolytic activity. Thus, His120 was shown to be essential for LasA activity [24].

2.2. Fungi

Fungal aspartic proteases are able to cleave substrate with "Lys" in the P1 position. Sequencing and structural comparison suggest that two aspartic acid residues (Asp30 and Asp77) may be responsible for conferring this unique specificity [25].Estell *etal*,engineered the substrate specificity of rhizopus pepsin from *Rhizopus niveus* and demonstrated the role of Asp77 in the hydrolysis of the substrates with lysine in the P-1 position [25]. The primary structure of aspergillopepsin I from *Aspergillus saitoi* ATCC 14332 (now designated A. phoenicis) was deduced from the nucleotide sequence of the gene [26]. To identify the residue responsible for determining the specificity of aspergillopepsin I toward the basic substrates in the substrate-binding pocket, Asp76 was replaced with a Ser residue by SDM. The striking feature of this mutation was that only the trypsinogen activating activity of the enzyme was destroyed, suggesting the importance of the Asp76 residue in binding to basic substrates. To elucidate whether the processing of the pro-region occurs by autoproteolysis or by involving a processing enzyme, Tatsumi *et al.* changed Ser228 to Ala by SDM [27]. *Saccharomyces cerevisiae* cells harboring a recombinant plasmid with mutant Alp did not secrete active Alp into the culture medium. The yeast cells accumulated a protein of 44 kDa, probably a precursor of Alp(the 34-kDa mature Alp plus the 10-kDa pro-peptide), suggesting that autoproteolytic processing of the pro-region was occurring. Introduction of a disulfide bond by SDM is known to enhance the thermostability of a cysteine-free enzyme. Aqualysin I, a thermostable subtilisin-type protease from *Thermus aquaticus* YT-1, contains four Cys residues forming two disulfide bonds [28]. The primary structure of Alp showed 44% homology to that of aqualysin I, and sites for Cys substitutions to form a disulfide bond were chosen in the Alp based on this homology. Ser69, Gly101, Gly169, and Val200 were replaced by Cys in the mutant Alp. Both Cys69-Cys101 and Cys169- Cys200 mutant Alps were expressed in *S. cerevisiae*, and the enzymes were purified to homogeneity. The Cys169-Cys200 disulfide bond was shown to increase the thermostability as well as the thermotolerance of *Aspergillus oryzae* Alp [29]. In vitro mutation of an aspartic acid residue predicted to be in the active site abolished the barrier activity of *S. cerevisiae* [30].

[III] DIRECTED EVOLUTION OF ENZYMES

This approach recently emerged as a key technology to generate enzymes with new and/ or improved properties that are extremely important for industrial applications [31]. This approach has provided a powerful tool for the development of enzymes with novel properties, even without requiring knowledge of enzyme structure and catalytic mechanisms. Jaeger *etal*, Amold *etal*, Petrounia *etal*, [31, 32, 33] have reviewed various aspects and examples of DE approaches for studying key properties of biocatalysts. The DE approach uses three approaches: DNA shuffling, random priming recombination and the staggered extension process (StEP). The DE starts with identification of a target enzyme followed by cloning of the corresponding gene. An efficient expression system is required before the target gene is subjected to random mutagenesis and/or in vitro recombination, thereby creating molecular diversity. This is followed by screening and identification of enzyme variants with the desired properties. However, use of the DE approach is still far from being widespread or adopted by many research laboratories using standard molecular biology techniques, as it is still unclear at present which strategy is the most efficient for the evolution of a desired property for a given protein [31]. However, some

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successful attempts have been made to evolve selective biocatalysts, such as lipase, aldolase, hydantoinase, esterase, aspartate aminotransferase, kanamycin nucleotidyl transferase, β-lactamase, β-galactosidase peroxidase, amidase and fucosidase, using the DE approach [31, 32]. Among the few reports on alkaline protease, subtilisin is the enzyme of choice for improving the catalytic behaviour of enzymes, using the DE approach. The total activity and organic solvent stability of subtilisin E from *B. subtilis* has been enhanced in aqueous dimethylformamide using the DE approach [34]. To demonstrate the utility of DE, Zhao *et al*,[35] used the StEP process to recombine a set of five thermostabilized subtilisin E variants identified during a single round of error-prone PCR mutagenesis and screening. Screening the StEP-recombined library yielded a subtilisin variant whose half-life was increased 50-fold at 50 °C, compared with wild-type subtilisin. In comparison, Ran et al, [36] reported the used of DE approach, using in vitro random mutagenesis and an improved screening method, to develop a cold-adapted mutant subtilisin from *B. subtilis* UOT0999 with a catalytic efficiency at 10 °C 100% higher than the wild-type strain. In another report, the thermal stability of subtilisin E was increased by converting it into a thermitase using DE [37]. This fascinating area of protein design will no doubt be the heart of future commitments in the enzyme industry.

3.1. A Flow Cytometry-Based Screening System for Directed Evolution of Proteases

Protein engineering, especially directed evolution, is a powerful tool and significant approach for protease reengineering. Directed protease evolution experiments produced proteases with improved catalytic efficiency such as cold adaption, increased thermostability, higher resistance toward oxidizing agents, activity in the absence of calcium ions, organic solvent resistance, and altered substrate specificity [38]. Some few reports employ protease inhibitors to probeactive sites of proteases to understand protease specificity [39, 40] or to access the potential of inhibitors as probable therapeutics [41].

Directed protease evolution campaigns conventionally use traditional screening formats such as agar plate (halo formation) or microplate-based detection systems[42]. The agar plate assays are usually applied in cases for a prescreening to identify the proteolytic activities at simple reaction conditions. The microplate-based screening methods are commonly used in directed evolution with capacities to be applied for more complex reaction cases. The two systems are usually associated with low mutational loads (one to three amino acid changes per mutated gene) so that a medium throughput is sufficient to find improved variants [43]. A notable example for a high-throughput screening (HTS) of protease variants is the directed evolution of the surface membrane protease OmpT to alter its substrate specificities by employing an *Escherichia coli* OmpT-deficient strain (UT5600)[44]. Improved variants were identified using flow cytometry in aqueous solution without employing any compartmentalization technology.

Miller *etal*, [45] reported recently, a (water-in-oil-in-water) double-emulsion-based compartmentalized flow cytometry screening technology (in vitro compartmentalization, IVC) has emerged as a potentially powerful screening format for sampling mutant libraries with approximately up to 108 variants per day. The IVC systems use a man made cell compartmentalization (e.g., w/o/w double-emulsion droplets), in which enzymatic reactions can be performed individually in femtoliter volume scale, resulting in a significant decrease of consumable cost. The principle of IVC-based flow cytometry screening systems employs fluorescence detection of a fluorescent product derived from a fluorogenic substrate. Therefore, the existence of a retainable fluorescence signal and its intensity are key parameters for the successful implementation of flow cytometry analysis employing IVC technologies [46]. In contrast to the surface adsorption method, the compartmentalized screening technology (IVC) is more generally applicable and could enable novel evolution strategies, for instance, by employing libraries with high mutational loads. Progress in IVC technology has been summarized well in several reviews [47, 48].

Ran *et al*, [38] created a first protease cytometry screening system (pro FC-IVC) which was developed by employing subtilisin Carlsberg as a model protease. Optimized parameters comprised (a) substrate selection to avoid partitioning of substrate and product into the oil phase, (b) host strain selection to minimize protease background reactions, and (c) host strain recovery after sorting. The developed protocol was subsequently validated by screening an epPCR library in which the mutational load was adjusted to 2%–3% of active clones.

In summary, Flow cytometry screening technology based on in vitro compartmentalization in double emulsion had been developed and applied on directed evolution of paraoxonase and β-galactosidase. In addition, advancements of flow cytometry– based screening technologies will enable an ultra-high throughput of variants offering novel opportunities in directed enzyme evolution under high mutational loads. For the industrially important enzyme class of proteases, a first flow cytometry–based screening system for directed protease evolution has been developed based on an extracellular protease-deficient *Bacillus subtilis* strain (WB800N), a model protease (subtilisin Carlsberg), and a water-in-oil-in-water double-emulsion technology. *B. subtilis* WB800N cells are incoporated in double emulsion with a fluorogenic substrate (rhodamine 110–containing peptide), paving way for the screening of protease variants in femtoliter compartments at high throughput. The protease screening technologyhas been validated by employing an epPCR mutant library with a high mutational load and screened for increased resistance toward the inhibitor antipain dihydrochloride. A variant (K127R, T237P, M239I, I269V, Y310F, I372V) with an improved

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relative resistance was isolated from a small population of active variants, validating the reported protease flow cytometry screening technology for increased inhibitor resistance [38].

[IV] ENZYMES ENGINEERING AND THEIR STABILITY IN SOLVENT

Reasearches all over the world for the past nearly three decades have unfolded the fact that, hostile environment provided to enzymes such as organic solvents, can catalyze reaction that were impossible in water, become more stable and exhibit new behavior such as "molecular memory". The proteins and/or enzymes stability is a major concern for their applications at industrial scales. High thermostable biocatalysts have a prolonged viability. For many biocatalysts, enzymatic reactions and stability of enzyme at high temperatures are pre-requisites for industrial use. However, the stability of enzyme in organic solvents, at extreme pH, pressure and stability towards mechanical disturbances are required for organic synthesis, chemical analysis, isolation and purification of chemicals, in therapeutics and diagnostics and in the study of protein structures and functions [36].

There has been persistent scientific effort to search for methods to prepare stable enzymes. Some of the examples of methods used for stabilizing microbial proteases are chemical modification using PEG, bio-imprinting of alpha (α) chymotrypsin in anhydrous media, chemical cross linking, molecular imprinting, immobilization in hydrophobic solvents, use of lyoprotectants [such as sugars, substrate-resembling ligands and crown ethers and protein engineering [36]. Inspite of the large number of research papers published on the organic-solvent stability of microbial proteases and peptide synthesis, only a few chosen methods have been adopted at industrial level. The primary reason being in ability of the rejected biocatalyst to have either of these two properties: substrate specificity, or sufficient organic-solvent stability. Thus, the discovery of novel microbial proteases having all the necessary required properties is considered extremely important for the use of enzymes on an industrial scale.

[V] CONCLUSIONS

Microbial proteases have numerous industrial and pharmaceutical applications. These applications are primarily achieved by engineering the enzymes using different approaches. The commercial successes of these enzymes lead to extensive study of biochemical, regulatory and molecular aspect of the enzymes system [26, 42]. The researchers have been and will continue to aim the discovery and engineering of novel microbial proteases that can perform efficiently at industrial scale. Currently, protein engineering of microbial proteases has been playing an important role in several industries. Different approaches such as SDM and DE will offer possibilities of generating proteases with entirely new function. The per suit for other newer approaches and/or strategies targeting new dimensions of molecular diversity and technology to improve performance characteristics by in vitro evolutionary changes of protein primary structures and high through put screening methods will continue to be the significant field of development in next few years.

REFERENCES

[1] Cooper JB, G Khan, G Taylor, IJ Yickle, TL Blundell. [1990] X-ray analyses of aspartic proteinases. II. Three dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 A resolution. *J. Mol. Biol.* 214:199–222.

[2] Pickersgill RW, IG Sumner, PW Goodenough. [1990]. Preliminary crystallographic data for protease a. *Eur. J. Biochem*. 190:443–444.

[3] Sobek H, HJ Hecht, B Hoffman, W Aeble, D Schomburg. [1990] Crystal structure of an alkaline protease from Bacillus alkalophilus at 2.4 A resolution. *FEBS Lett*. 274:57–66.

[4] Thomas PG, AJ Russell, AR Fersht. [1985] Tailoring the pH dependence of enzyme catalysis using protein engineering. *Nature* 28:375– 376.

[5] Takagi H, T Morinaga, H Ikemuar, M Inouye. [1989]. The role of pro-239 in the catalysis and heat stability of subtilisin E. *Eur. J Biochem.* 105:953–956.

[6] Takagi H, Y Morinaga, H Ikemura, M Inouye. [1988] Mutant subtilisin E with enhanced protease activity obtained by site directed mutagenesis. *J Biol. Chem.* 263:19592–19596.

[7] Imanaka T, M Shibazaki, M Takagi. [1986] A new way of enhancing the thermostability of proteases. *Nature* 324:695–697.

[8] Jacobs M, M Eliasson, M Uhlen, and JI Flock. [1985] Cloning, sequencing and expression of subtilisin Carlsberg from Bacillus licheniformis. *Nucleic Acids Res.* 13:8913–8926.

[9] Ballinger MD, J Tom, and JA Wells. [1996] Furilisin: a variant of subtilisin BPN9 engineered for cleaving tribasic substrates. *Biochemistry* 35:13579–13585.

[10] Berglund P, MR Stabile, M Gold, JB Jones, C Mitchinson. [1996] Altering the specificity of subtilisin B. lentus by combining site-directed mutagenesis and chemical modification. *Bioorg. Med. Chem. Lett*. 6:2507–2512

[11] Carter P, and JA Wells. [1987] Engineering substrate specificity by substrate assisted catalysis. *Science* 237:394–399.

[12] Estell DA, TP Gyaycar, JV Miller, DB Powers, JP Burnier. [1986]. Probing steric and hydrophobic effects on enzyme substrate interactions by protein engineering. *Science* 233:659–663.

[13] Russel AJ, and AR Ferhst. [1987] Rational modification of enzyme catalysis by engineering surface charge. *Nature* 328:496–500.

[14] Mitchinson C, and JA. Wells. [1989] Protein engineering of disulfide bonds in subtilisin BPN9. *Biochemistry* 28:4807–4815

[15] Pantoliano MW, RC Ladner, PN Bryan, ML Rollence, JF Wood, TL Poulos. [1987] Protein engineering of subtilisin BPN9: enhanced stabilization through the introduction of two cysteines to form a disulfide bond. *Biochemistry* 26:2077–2082.

[16] Stabile MR, WG Lai, G DeSantis, M Gold, and JB Jones. [1996].Probing the specificity of the S1 binding site of M222 mutants of subtilisin B. lentus with boronic acid inhibitors. *Bioorg. Med. Chem. Lett.* 6:2501– 2506.

[17] Wells JA, and DB Powers. [1986] In vivo formation and stability of engineered disulfide bonds in subtilisin. *J Biol. Chem.* 261:6564–6570.

[18] Takagi H, T Takahashi, H Momose, M Inouye, Y Maeda, H Matsuzawa, and T Ohta. [1990] Enhancement of the thermostability of subtilisin E by introduction of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease. *J Biol. Chem.* 265:6874– 6878.

[19] Strausberg SL, PA Alexander, DT Gallagher, GL Gilliland, BL Barnett, and PB Bryan. [1995] Directed evolution of a subtilisin with calcium-independent stability. *Bio Technology* 13:669–673.

[20] Eijsink VGH, JK. vander Zee, B vanden Burg, G Vriend, and G Venema. [1991] Improving the thermostability of the neutral protease of Bacillus stearothermophilus by replacing a burried aspargine by leucine. *FEBS Lett*. 282:13–16.

[21] Eijsink VGH, G Vriend, B vanden Burg, JR vander Zee, and G Venema. [1992]. Increasing the thermostability of a neutral protease by replacing positively charged amino acids in the N-terminal turn of α-helices. *Protein Eng*. 5:165–170.

[22] Bruinenberg PG, WM De Vos, and RJ Siezen. [1994] Prevention of C-terminal autoprocessing of Lactococcus lactis SK11 cell-envelope proteinase by engineering of an essential surface loop. *Biochem. J* 302:957– 963.

[23] Graham LD, Haggett PA, Jennings DS. Brocque, and RG Whittaker. [1993] Random mutagenesis of the substrate binding site of a serine protease can generate enzymes with increased activities and altered primary specificities. *Biochemistry* 32:6250–6258.

[24] Gustin JK., E Kessler, DE Ohman. [1996] A substitution at His-120 in the LasA protease of Pseudomonas aeruginosa blocks enzyme activity without affecting propeptide processing or extracellular secretion. *J Bacteriol* 178:6608–6617.

[25] Lowther WT, P Majer, BM Duhn. [1995] Engineering the substrate specificity of rhizopuspepsin: the role of Asp77 of fungal aspartic proteinases in facilitating the cleavage of oligopeptide substrates with lysine in P1. *Protein Sci.* 4:689–702.

[26] Rao MB, Tanksale AM, Ghatge MS. et al. [1998] Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev* 62:597–635.

[27] Tatsumi HS. Murakami Y, Ogawa A, Masaki Y, Ishida K, et al.[1991]. Autoproteolytic processing and processing dependent secretion of Aspergillus oryzae protease in yeast. Agric. *Biol. Chem.* 55:3099–3101.

[28] Kwon ST, H Matsuzawa, T Ohta. [1988] Determination of the positions of the disulfide bonds in aqualysin I (a thermophilic alkaline serine protease) of Thermus aquaticus YT-1. *J. Biochem.* 104:557–559.

[29] Ikegaya KY. Ishida K, Murakami A, Masaki N, Sugio K, et al. [1992]. Enhancement of the thermostability of the alkaline protease from Aspergillus oryzae by introduction of a disulfide bond. Biosci. Biotechnol. *Biochem*. 56:326–327.

[30] MacKay VL, SK Welch, MY Insley, TR. Manney, J Holly, GC Saari, and ML Parker. [1988]. The Saccharomyces cerevisiae BAR1 gene encodes an exported protein with homology to pepsin. *Proc. Natl. Acad. Sci.* USA 85:55–59.

[31] Jaeger KE, Eggert T, Eipper A, et al. [2001] Directed evolution and the creation of enantioselective biocatalysis. *Appl Microbiol Biotechnol* 55:519–530 .

[32] Arnold FH, [1996] Directed evolution: creating biocatalysts for the future. *Chem Eng Sci* 51:5091–5102

[33] Petrounia IP, Arnold FH. [2000] Designed evolution of enzymatic properties. *Curr Opin Biotechnol* 11:325–330.

[34] You L, Arnold FH, [1996] Directed evolution of subtilisin E in Bacillus subtilis to enhance total activity in aqueous dimethylformamide. *Protein Eng* 9:77–83.

[35] Zhao H, Giver L, Shao Z, et al. [1998] Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nature Biotechnol* 16:258

[36] Gupta R, Beg QK, Lorenz P. [2002] Bacterial alkaline proteases: Molecular approaches and industrial applications. *Appl Microbiol Biotechnol Rev* 59:15–32

[37] Zhao H, Arnold FH, [1999]. Directed evolution converts subtilisin E into a functional equivalent of thermitase. *Protein Eng* 12:47–53

[38] Ran T, Ronny M, Radivoje P. etal. [2011] A Flow Cytometry Based Screening System for Directed Evolution of Proteases. *J Biomol Screen* 16: 285

[39] Storer AC. [1991] Engineering of Proteases and Protease Inhibition. *Curr. Opin. Biotechnol* 2: 606–613.

[40] Laskowski M, Qasim MA. [2000] What Can the Structures of Enzyme-Inhibitor Complexes Tell Us about the Structures of Enzyme Substrate Complexes? *Biochim. Biophys. Acta.,* 1477: 324–337.

[41] Turk, B. Targeting Proteases: Successes, Failures and Future Prospects [2006]. Nat. Rev. Drug Discov., 5, 785–799.

[42] Gupta R, Beg QK, Khan S, Chauhan B. [2002] An Overview on Fermentation, Downstream Processing and Properties of Microbial Alkaline Proteases. *Appl. Microbiol. Biotechnl,* 60: 381–395.

[43] Wong TS, Zhurina D, Schwaneberg U. [2006] The Diversity Challenge in Directed Protein Evolution. Comb. Chem. High Throughput Screen. 9: 271–288.

[44] Varadarajan N, Rodriguez S, Hwang BY, Georgiou G, Iverson BL. [2008] Highly Active and Selective Endopeptidases with Programmed Substrate Specificities. *Nat. Chem. Biol*. 4: 290–294.

[45] Miller OJ, Bernath K, Agresti JJ, Amitai G, Kell BT, Mastrobattista E, Taly V, Magdassi S, Tawfik DS, Griffiths AD. [2006] Directed Evolution by In Vitro Compartmentalization. *Nat. Methods* 3: 561–570.

[46] Bernath K, Hai M, Mastrobattista E, Griffiths AD, Magdassi S, Tawfik DS. [2004] In Vitro Compartmentalization by Double Emulsions: Sorting and Gene Enrichment by Fluorescence Activated Cell Sorting. *Anal. Biochem* 325: 151–157.

[47] Yang G, Withers SG. [2009] Ultrahigh-Throughput FACS-Based Screening for Directed Enzyme Evolution. *Chembiochem* 10: 2704–2715.

[48] Griffiths AD, Tawfik DS. [2006] Miniaturising the Laboratory in Emulsion Droplets. *Trends Biotechnol* 24: 395–402.

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RESEARCH ARTICLE OPEN ACCESS

EFFECTS OF SALICYLATE ON GROWTH AND BIOCHEMICAL CHANGES IN MAIZE SEEDLINGS UNDER SALT STRESS

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ABSTRACT

Protective effects of exogenous salicylic acid (SA) on maize (Zea mays L.) seedlings under salinity stress were studied. Pre-soaking treatments of NaCl (0, 50, 100, 150 and 200mM) were given to maize seeds in presence as well as absence of 0.5 mM salicylic acid. The injurious effects of salinity on growth and development were manifested by decreased dry weight, leaf area, number of roots, and percentage water content along with reduction in biochemical components. Activity of both superoxide dismutase (SOD) (EC 1.15.1.1) and catalase (CAT) (EC 1.11.1.6) increased during saline conditions, however SA pretreatment increased SOD activity further and the activity of catalase was decreased in antagonistic manner. Degree of lipid peroxidation declined through the significant reduction in MDA content in maize seedlings. Results suggests that exogenous Salicylic acid reduced the detrimental effects of salinity and controlled growth, development and stress responses in maize plants.

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KEY WORDS

Antioxidant enzymes; Lipid peroxidation; Salicylic acid; Salinity; Zea mays

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[I] INTRODUCTION

Salt stress is one of the major problems for agriculture as it prevents plants from realizing their yield potential and has become more prevalent as the intensity of agriculture increases [1]. Salinity stress-induced metabolic changes are enhanced due to accumulation of toxic compounds in cells that include excess ions and reactive oxygen species (ROS). ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, and peroxidases [2] and /or involved in oxidative signal transduction which in turn trigger the antioxidant defense system associated with the mechanisms by which plant cells sense the environment and make appropriate adjustments to gene expression [3], metabolism [4] and physiology [5]. Development of methods to induce salt stress tolerance in plants is vital and receives considerable attention to increase plant productivity.

Salicylic acid (SA) has appeared as a new phytohormone biosynthesized from the phenylalanine in plant metabolism and considered as an important signaling molecule modulating plant responses to abiotic stresses [6]. Studies have shown that plant dry mass under abiotic stresses was significantly higher with the application of SA than without SA [7, 8]. However, effects of exogenous SA on physiology and biochemistry of plant species under saline condition are still not understood.

In the present work it is demonstrated that SA pretreatment caused protection against salinity in 2-week-old maize seedlings under saline and non-saline conditions.

[II] MATERIALS AND METHODS

Seeds of maize (Zea mays L.) var. Jaunpuri were procured from Plant Breeding Department, Institute of Agricultural Sciences, B.H.U., India. Seeds were surface sterilized with 0.01% $HgCl_2$ followed by thorough washing with glass-distilled water. Homogenous lots of surface sterilized seeds were presoaked in different treatments for 6 h as follows: Distilled water (Control); 0.5 mM SA; 50, 100, 150 and 200 mM of NaCl and 0.5mM SA with each level of salinity. Treated seeds were placed on moistened Whatman no. 1 filter paper in acid washed Petri dishes in dark at 27°C for germination and thereafter transferred to acid washed sand in polybags. Plants were grown in a glasshouse under natural light conditions (in range of 27-35° C air temperature, 450-500 µmol/m2 s light intensity and 75% relative humidity). Each polybag contains 6 plants and supplied with 20 ml of 50% of Hoagland's nutrient solution at alternate days [9].

Plants were harvested two weeks after the germination and dried in a thermoventilated oven at 70ºC until constant weight. Growth parameters like number of roots, % water content, leaf area and dry weight were calculated according to the standard methods. Various biochemical analyses were performed in the leaf samples of two weeks old maize plants. Pigment contents,soluble protein and total phenolic content were estimated as per Lichtenthaler [10], Lowry *et al*. [11] and Farkas and

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Kiraly [12] respectively. MDA content, Catalase activity and Superoxide dismutase activity were determined as per Heath and Packer [13], Kar and Mishra [14] and Beauchamp and Fridovich [15] respectively. The experiments were repeated twice with three replicates (n=5) and the data presented are mean ± standard errors (SE). The results were subjected to one-way ANOVA and means were compared by the least significant difference (LSD) test and Tukey's multiple range test at the 0.05 and 0.01 percent level of significance.

[III] RESULTS AND DISCUSSION

Plant growth: Growth pattern of maize plants was adversely affected with exposure to NaCl salinity. 0.5 mM SA application resulted in significant increase in dry matter yield both in saline and non-saline conditions **[SupplementaryTable–1]**, however, effect was more pronounced under saline condition as compared to non-saline conditions. Shakirova *et al*. [16] also reported similar results. Increase in dry matter of salt stressed plants in response to SA may be attributed to antioxidants and protective role of membranes that alleviate the plants tolerance to damage.

Pigments, soluble protein and phenolics: Data presented in **Supplementary Table–2**. show that pigments content of NaCl-treated maize plants was significantly lower compared to controls. Whereas, in presence of 0.5 mM salicylic acid, effects of NaCl-stress on the pigment content was reduced upto 50%. Soluble protein content decreased sharply with increasing concentrations of NaCl-stress **[Supplementary Table–2]**. Retardation in soluble protein content was significantly observed at 150 mM, reaching maximum at 200 mM of salt stress. The reduction in the level of soluble protein content under salinity may be due to breakdown/ degradation of chlorophylls or due to inhibition of foliar proteins required for the genesis of photosynthetic pigments. While, in presence of 0.5mM salicylic acid effects of NaCl stress were counteracted and soluble protein levels were increased significantly. Salicylic acid is supposed to increase the functional state of photosynthetic machinery in plants either by the mobilization of internal tissue nitrate or chlorophyll biosynthesis [17]. This may lead to increased soluble protein. NaCl-stressed accumulation of total phenolics was very high than that of control (2 fold) in absence of salicylic acid. Phenolics constitute a part of cellular solutes and provide a reducing environment to the system [18]. Whereas, in the presence of exogenous salicylic acid, phenolics content was reduced significantly **[Supplementary Table–2]**. Salicylic acid modulates plant responses to a wide range of oxidative stresses [19].

Lipid peroxidation: Degree of lipid peroxidation was measured as accumulation of MDA content in leaf tissues of 2 weeks old maize seedlings. Accumulation of MDA in NaCl stressed (200mM) maize seedlings was very high (2 fold) than that of control in absence of SA, whereas in the presence of 0.5mM exogenous SA content of MDA was reduced significantly **[Figure–1]**. MDA content could reflect the degree of membrane lipid peroxidation with increasing concentrations of NaCl-stress because lipid peroxidation is caused by the reaction between •OH and the methylene groups of polyunsaturated fatty acids, which are the main components of membrane lipids [20]. Present findings are in agreement with those of Gunes *et al*. [21].

Antioxidant enzymes: ROS scavenging enzymes (SOD, catalase), activity increased with exposure to NaCl salinity (0, 50, 100,150, 200mM) in maize seedlings in the presence of 0.5 mM SA. In absence of SA, activity of catalase increased significantly while activity of superoxide dismutase (SOD) was found as good as control **[Figure– 2 A and B]**. After 0.5 mM of SA pretreatment a decrease in catalase activity was observed which exhibit the inhibitory properties of SA to catalase activity in several plant species [22]. In the present case a pronounced decrease was found in the catalase activity after 0.5 mM SA pretreatment to maize seeds. This decrease could be due to a secondary effect of SA rather than to a direct catalase-binding affinity resulting in enhancement of H_2O_2 production and subcellular localization in the cells [23]. Increasing NaCl (50-200 mM) treatments (14 days) increased SOD activity with respect to the control values. Moreover, application of 0.5 mM SA induced higher SOD activity than those of salt stressed maize plants. This implies that SA might be involved in the positive amplification loops in ROS signaling pathways, which results in enhanced production and amplification of the ROS signals [24]. Accumulation of ROS in cells might activate the ROS-scavenging pathways (leading to increasement in SOD and other antioxidant enzymes) and result in suppression of ROS [25]

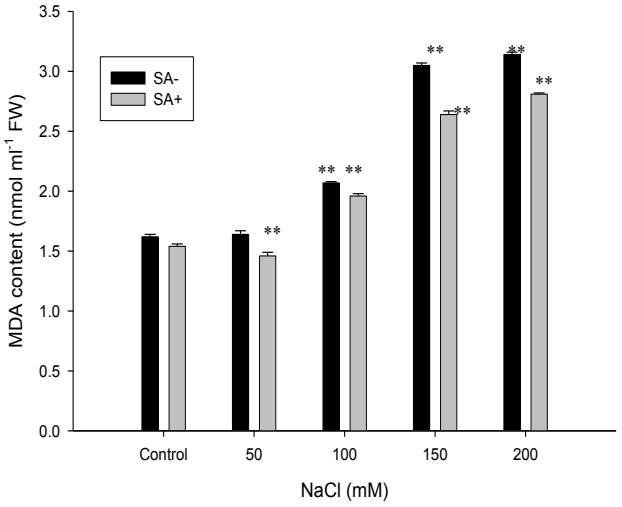

Fig: 1. Effects of salicylic acid on MDA content in 2-week-old maize seedlings under increasing concentration of NaCl. 0.5 mM SA was given as presoaking seed treatment. Data presented are mean ± S.E. (n=5). ** represent significant differences compared to controls at P < 0.01 according to Tukey's multiple range test. LSD values were determined at *P* < 0.05.

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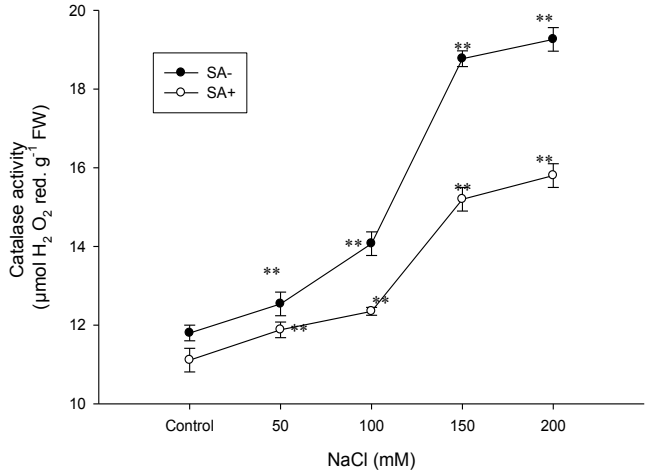

Fig: 2(A). Effects of salicylic acid on changes in (A) CAT and (B) SOD activity in 2- week-old maize seedlings under increasing concentration of NaCl. 0.5 mM SA was given as presoaking seed treatment. Data presented are mean ± S.E. (n=5). ** represent significant differences compared to controls at $P < 0.01$ according to Tukey's multiple range test. LSD values were determined at $P < 0.05$

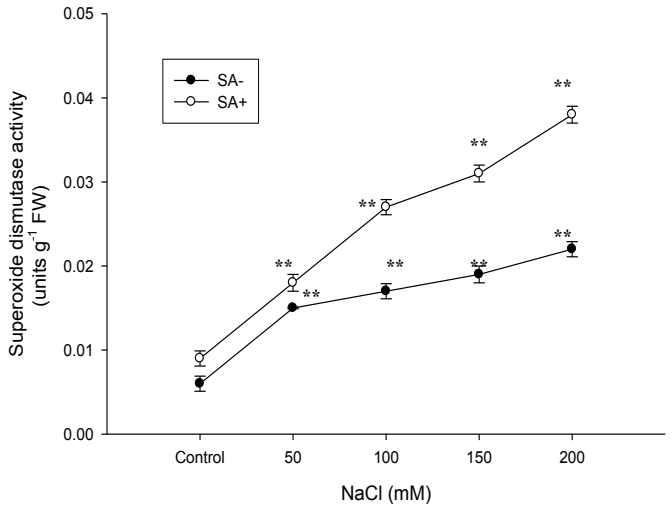

Fig: 2(B). Effects of salicylic acid on changes in (A) CAT and (B) SOD activity in 2- week-old maize seedlings under increasing concentration of NaCl. 0.5 mM SA was given as presoaking seed treatment. Data presented are mean ± S.E. (n=5).** represent significant differences compared to controls at P<0.01 according to Tukey's multiple range test. LSD values were determined at $P < 0.05$

[IV] CONCLUSION

In conclusion salinity induced oxidative stress in maize plants, as evidenced by the decline in growth, increase in lipid peroxidation and changes in antioxidant defence mechanism. The potentiating effect of SA was observed in present study after pretreating maize seeds with 0.5 mM SA, where the deleterious effects of salinity was restored as growth, lipid peroxidation and pattern of antioxidant enzymes in maize plants.

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REFERENCES

[1] Zhu JK. [2002] Salt and drought stress signal transduction in plants. Annu. Rev. *Plant Biol* 53: 247–273.

[2] Suzuki N, Mittler R. [2006] Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. *Physiol Plant* 126: 45–51.

[3] Sairam RK, Tyagi A. [2004] Physiology and molecular biology of salinity stress tolerance in plants. *Curr. Sci*. 86: 407–421.

[4] Kavi Kishor PB, Sangam S, Amrutha RN, SriLaxmi P, Naidu KR, *et al*. [2005] Regulation of proline biosynthesis, degradation, uptake and transport in higher plants : Its implications in plant growth and abiotic stress tolerance. *Curr Sci.* 88: 424–438.

[5] Foyer CH, Noctor G. [2005] Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. *Plant Cell* 17: 1866–1875.

[6] Senaratna T, Touchell D, Bunn T, Dixon K. [2000] Acetyl salicylic acid (Aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. *Plant Growth Regul* 30 : 157–161.

[7] Singh B, Usha K. [2003] Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress. *Plant Growth Regul*. 39: 137–141.

[8] Gautam S, Singh PK. [2009] Salicylic acid-induced salinity tolerance in corn grown under NaCl stress. *Acta Physiologia Plantarum*.31: 1185–1190.

[9] Hoagland DR, Arnon DI. [1950] The water culture method for growing plants without salt. California Agri. *Exp. Stat.Circ* 347:32

[10] Lichtenthaler HK. [1987] Chlorophylls and carotenoids: Pigments of photosynthetic biometers. *Methods Enzymol* 148: 350–382.

[11] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. [1951] Protein Measurement with the Folin Phenol Reagent. *J Biol. Chem* 193: 265–275.

THE IIOAB JOURNAL
ISSN: 0976-3104

[12] Farkas GL, Kiralay Z. [1962] Role of phenolic compounds in the physiology of plant disease and disease resistance. *Phytopath* 44: 105–150.

[13] Heath R, Packer L. [1968] Photooxidation in isolated chloroplast.1.Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125: 189–198.

[14] Kar M, Mishra D. [1976] Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. *Plant Physiol* 57: 315–319.

[15] Beauchamp C, Fridovich I. [1971] Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochem* 44: 276–287.

[16] Shakirova FM, Sakhabutdinova AR, Bezrukova MV, Fathutdinova RA, Fathutdinova DR. [2003] Changes in hormonal status of wheat seedlings induced by salicylic acid and salinity. *Plant Sci* 164: 317–322.

[17] Shi Q, Bao Z, Zhu Z, Ying Q, Qian Q. [2006] Effects of different treatments of salicylic acid on heat tolerance, chlorophyll fluorescence and antioxidant enzyme activity in seedlings of Cucumis sativa L. *Plant Growth Regul*. 48: 127–135.

[18] Das N, Misra M, Misra AN. [1990] Sodium chloride salt stress induced metabolic changes in callus cultures of pearl millet (Pennisetum americanum L.). *J Plant Physiol* 137: 244–246.

[19] Shirasu K, Nakajima H, Rajshekar K, Dixon RA, Lamb C. [1997] Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signal in the activation of defense mechanism. *Plant Cell* 9: 261–270.

[20] Blokhina OB, Fagerstedt KV, Chirkova TV. [1999] Relationship between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. Physiol. *Plant* 105: 625–632.

[21] Gunes A, Inal A, Alpaslan M, Eraslan F, Bagci EG. Cicek N. [2007] Salicylic acid induced changes on some physiological parameters symptomatic for oxidative stress and mineral nutrition in maize (Zea mays L.) grown under salinity. *J Plant Physiol* 164: 728–736.

[22] Rao MV, Paliyath G, Ormrod DP, Murr DP, Watkins CB. [1997] Influence of salicylic acid on H2O2 production, oxidative stress, and H2 O2–metabolizing enzymes. *Plant Physiol* 115: 137–149.

[23] Chen Z, Silva H, Klessig DF. [1993] Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262: 1883–1886.

[24] Mittler R, Vanderauwera S, Gollery M, Breusegem FV. [2004] Reactive oxygen gene network of plants. *Trends Plant Sci* 9: 490-498.

[25] Devi S, Angrish R, Datta KS, Kumar B. [2008] Antioxidant defence system in wheat seedlings under sodium chloride stress: An inductive role of hydrogen peroxide. *Indian J Plant Physiol* 13: 118–124.

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Supplementary Tables (As supplied by author)

Supplementary Table: 1. Effects of salicylic acid on growth characteristics of 2-week-old maize plants under NaCl stress

NaCl (mM)	Dry weight (g)		Leaf area (cm2)		No. of roots		Percentage water content	
	SA-	SA+	SA-	SA+	SA-	SA+	SA-	SA+
0	2.97 (±0.66)	3.56 (±0.80)	28.1 (±7.43)	42.2 (±8.26)	14 (±3.57)	19 (±4.03)	75.58 (±16.90)	76.28 (±17.05)
50	2.56** (±0.57)	3.09** (±0.69)	23.2** (±5.80)	40.5** (±7.63)	11** (±2.91)	17 (±3.57)	78.68** (±17.60)	78.77* (±17.42)
100	2.18** (±0.49)	2.69** (±0.60)	18.7** (±5.24)	34.4** (±6.37)	8** (±2.24)	14** (±2.91)	78.56** (±17.50)	78.96** (±17.96)
150	1.90** (±0.42)	2.09** (±0.47)	12.2** (±4.50)	30.1** (±5.73)	6 (±1.79)	12 (±2.46)	76.66** (±17.10)	82.54** (±18.46)
200	1.54** (±0.34)	2.00** (±0.45)	6.1** (±2.41)	24.2** (±4.09)	3** (±1.12)	10** (±2.01)	75.77** (±16.90)	78.74** (±17.61)
LSD	(0.019)	(0.021)	(0.022)	(0.023)	(1.072)	(1.433)	(0.028)	(0.023)

SupplementaryTable: 2. Effects of salicylic acid on changes in biochemical components of 2-week-old maize plants under NaCl stress

NaCl (mM)	Total chlorophyll (mg g^{-1} FW)		Carotenoids (mg g^{-1} FW)		Soluble protein (mg g^{-1} DW)		Total phenolics (mg g^{-1} FW)	
	SA-	SA+	SA-	SA+	SA-	SA+	SA-	SA+
0	4.15 (±0.92)	7.19 (±1.61)	1.84 (±0.41)	2.98 (±0.44)	1.44 (±0.32)	1.91 (±0.43)	0.121 (±0.03)	0.127 (±0.03)
50	3.33** (±0.74)	6.56** (±1.47)	1.36** (±0.30)	2.57** (±0.34)	1.07** (±0.24)	1.82** (±0.41)	0.128** (±0.03)	0.132 (±0.03)
100	1.82** (±0.41)	5.71** (±1.27)	0.90** (±0.23)	2.23** (±0.28)	1.00** (±0.22)	1.34** (±0.29)	0.151** (±0.03)	0.134 (±0.03)
150	0.81** (±0.18)	4.88** (±1.09)	0.70** (±0.18)	2.01** (±0.23)	0.73** (±0.16)	1.09** (±0.24)	0.199** (±0.04)	0.169** (±0.04)
200	0.28** (±0.06)	3.73** (±0.83)	0.50** (±0.17)	1.81** (±0.21)	0.54** (±0.12)	0.98** (±0.22)	0.237** (±0.05)	0.179** (±0.04)
LSD	(0.020)	(0.021)	(0.020)	(0.015)	(0.017)	(0.014)	(0.0001)	(0.020)

Data presented are mean ± SE (n=5). * and ** represent significant differences compared to controls at $P < 0.05$ and $P < 0.01$ respectively according to Tukey's multiple range test LSD values were determined at $P < 0.05$

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RESEARCH ARTICLE OPEN ACCESS

A CASE OF CENTRIC FUSION TRANSLOCATION IN A DEONI (*BOS INDICUS*) INDIAN CATTLE BULL CALF

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ABSTRACT

Introduction: *Translocations are very common in cattle This is the first report on the Robertsonian translocation in phenotypically normal Deoni cattle bull calf out of 458 cattle breeds of Indian origin Bos indicus screened so far to detect chromosomal aberrations during routine investigation.* ***Objective:*** *Detection of chromosomal aberrations in breeding bulls as the aberrations are associated to fertility problem in domestic animals.* ***Method:*** *Lymphocyte culture was set in a growth medium, RPMI-1640 supplemented with fetal calf serum, antibiotics, and mitogen. Culture was incubated at 37 degree C for 72 hrs and metaphase was arrested for chromosome study.* ***Results:*** *All metaphase chromosomes exhibited 59 chromosomes instead of 60. Cytogenetic investigation revealed the less chromosome numbers are because of centric fusion of two chromosomes; probably involving chromosome number 16 and 20. Conclusion: This is the first report of Deoni cattle (Bos indicus) as usually translocation or centric fusions are common chromosomal aberrations of Bos taurus cattle. Animals with centric fusion may not be used for artificial programme as the abnormality can cause repeat breeding problem in breedable female population.*

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KEY WORDS

Bos indicus; chromosome; translocation; centric fusion; autosomes

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[I] INTRODUCTION

Among various numerical and structural chromosomal aberrations reported so far, the translocations are very common in cattle breeds, which are known to cause varying degrees of subfertility (Long, 1985). In balanced form, it has no visible effect on body conformation because the genetic material present in the centric fusion chromosome is the same as in the two separate chromosomes. The centric fusion translocations are also known as Robertsonian translocation as it was first observed by W R B Robertson [1] while working with grasshoppers. The Robertsonian 1/29 translocation is the most frequent structural chromosomal abnormality in cattle [2] that was first observed in Swedish Red breed of cattle [3]. It has been documented in various frequencies in about 60 different breeds of both *Bos taurus* and *Bos indicus* [4]. Besides, many cases of centric fusion translocation involving other chromosomes have also been reported in various breeds of cattle worldwide [5, 6, 7, 8, 9, 10, 11, 12]. Such translocations have also been documented for other large animal species [13, 14].

India possesses 15% of the total cattle population and there are 30 distinct breeds of indigenous cattle (National Bureau Animal Genetic Resources, India). These cattle are distributed in three categories, mainly milch, drought and dual purpose breeds. In addition, a large number of non-descript cattle with poor productivity are also geographically distributed all over the country. However, most of Indian cattle breeds are well known for their draught resisting quality and to with stand diseases and parasites. Deoni is one of Indian cattle breeds (*Bos indicus*) known as dual purpose cattle.

This is the first time that a centric fusion translocation appeared in an Indian *Bos indicus* breed that was encountered during routine cytogenetic investigation prior to selection of bull for semen production.

[II] MATERIALS AND METHODS

Peripheral blood was collected from phenotypically normal 2 years old young bull calf of Deoni breed, in a heparinzed vacutainer blood collecting tube. Chromosomal preparations were performed by using standard whole blood culture in RPMI-1640 (Gibco) medium supplemented with antibiotics,15% fetal calf serum and 1% pokeweed mitogen [12]. The blood culture was incubated at 37°C for 72 hours. To increase the relative frequency of prometaphase chromosomes, Ethidium bromide (Sigma) @10 µg/ml was added and to arrest somatic cell division at metaphase stage, Colchicine (Sigma) @ 2 µg/ml was added to the culture for 2 and 1 h respectively, prior to the harvesting. The cells were separated by centrifugation at 150 g for 5 minutes followed by hypotonic treatment with 0.56% KCl for 20 minutes at 37°C and fixed in 3:1 ratio of methanol and acetic acid glacial. Finally, cell suspension was dropped on slides and air dried. Slides were conventionally stained in Giemsa stain for screening under the Nikon

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compound microscope attached with photographic system.

[III] RESULTS AND DISCUSSION

The cattle normally possess 60 (2n) chromosomes. The karyotype composed of 29 pairs of autosomes and one pair of sex chromosomes. All the autosomes are acrocentric and sex chromosomes (XY) are submetacentric in Bos taurus, whereas Y chromosome in *Bos indicus* is acrocentric. In the present case, all the scored 50 metaphase plates of the bull exhibited a diploid number of 59 due to presence of a biarmed chromosome, in addition to the submetacentic X and acrocentric Y chromosomes [Figure–1]. This is the first time that a centric fusion translocation appeared in Deoni breed out of 458 different breeds of *Bos indicus* screened during routine investigation (karyotyping). This finding is similar to many cases reported earlier [15, 16, 17] wherein they reported 16/20 translocation. As compared to exotic cattle, no case of translocation is reported in *Bos indicus* in India. However, a few cases of translocations were reported in Indian Jersey and Holstein crossbred cattle, and buffalo population. Thiagrajan et al. [18] identified 1/29 translocation in an Indian Jersey crossbred heifer with the history of anoestrus. Similarly, Chauhan et al [19] have also observed 1/29 translocation in a Jersey crossbred bull calf. Patel [12] reported a new centric fusion translocation [7, 16] in an Indian Holstein crossbred bull. Two cases of unusual translocation were also reported in Murrah buffaloes [20, 21] in India. Except unusual cases [19], the fertility of male is not grossly affected because of centric fusion.

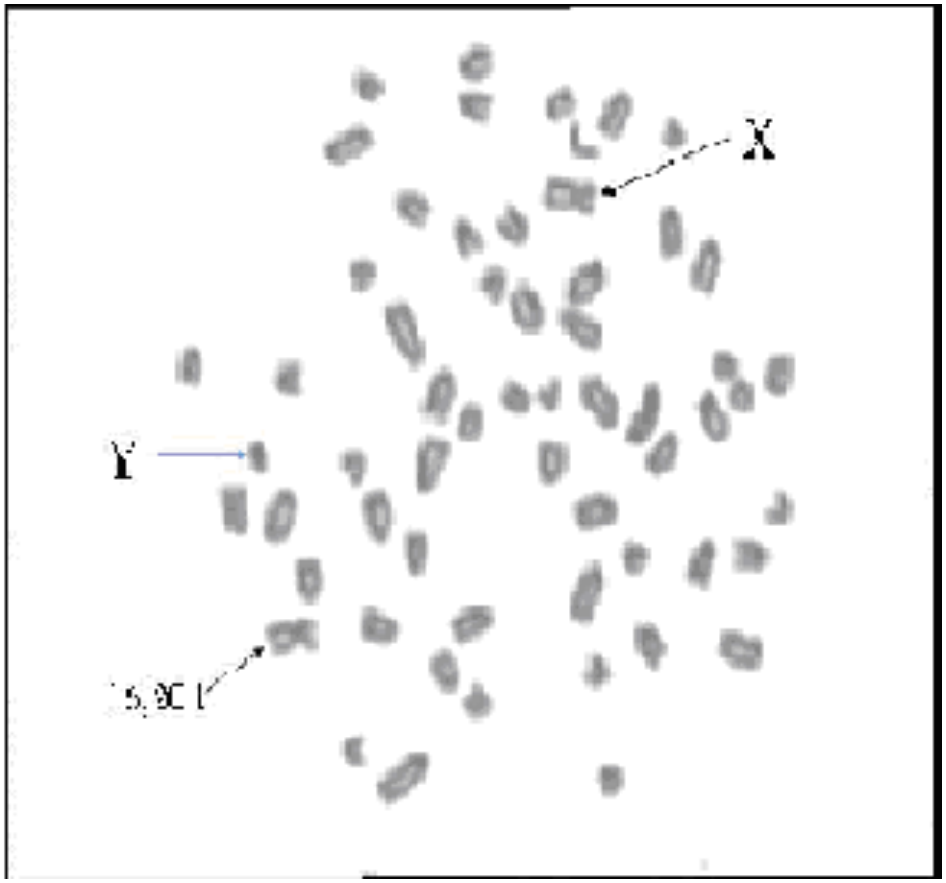

Fig. 1: Metaphase plate of *Bos indicus*

The reproductive potential of Deoni bull calf with 16/20 translocation in present study is not available since it is not in semen production and it was immediately culled from the semen station because of chromosomal aberration. It is always advisable to cull and avoid using such bulls for semen production as the Robertsonian translocation can have an adverse effect on fertility, apparently due to the production of chromosomally unbalanced gametes [22, 5]. Chromosome analysis of embryos indicated the occurrence of trisomic embryos resulting from the fertilization of normal ova by hyperhaploid spermatozoa [23]. Such unbalanced zygotes which tend to die at an early stage of development in females thus giving repeat breeding problems in normal females [24, 25].

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REFERENCES

[1] Robertson WRB. (1916) Chromosome studies I. Taxonomic relationship shown in the chromosomes of Tattigidae and Acrididae. Louustidae and Gryllidae: Chromosomes and Variation. *J Morph* 27:179–331.

[2] Popescu CP. (1978) A study of meiotic chromosomes in bulls carrying the 1/29 translocation. *Ann. Biol. Anim Bioch Biophys* 18: 383–389.

[3] Gustavsson I, Rockborn G. (1964) Chromosome abnormality in three cases of lymphocytic leukaemia in cattle. *Nature* 203:990.

[4] Popescu CP, Pech A. (1991) Une bibliographie Sur la translocation 1/29 de bovins dans le monde (1964- 1990). *Ann Zootech* 40:271–305.

[5] Teteno H, Miyaka YI, Mori H, Kamiguchi Y, Mikamo K. (1994) Sperm chromosome study of two bulls heterozygous for different Robertsonian translocations. *Hereditas* 120:7–11.

[6] Berland HM, Sharma A, Cribiu EP, Darre R, Boscher J, Popescu CP. (1988) A new case of Robertsonian ttranslocation in cattle. *J Heredity* 79:33–35.

[7] Pinheiro LEL, Carvalho TB, Oliveira DAA, Popescu CP, Basrur PK. (1995) A 4/21 tandom fusion in cattle. *Hereditas* 122: 99-102.

[8] Molteni L, Giovanni-Macchi A, De Succi G, Cremonesi F, Stacchezzini S, Di Meo GP, Iannuzzi L. (1998) A new centric fusion translocation in cattle: rob(13;19). *Hereditas* 129: 177–180

[9] Iannuzzi L, Range-Figueiredo T, Di Meo GP, Ferrara L. (1993) A new centric fusion translocation in cattle, rob (16;18). *Hereditas* 119: 239-243.

[10] Iannuzzi L, Rangel-Figueiredo T, Di Meo GP, Ferrara L. (1992) New Robertsonian translocation in cattle, rob (15;25). *Cytogenet Cell Genet* 59:280–283.

[11] Vallenzasca C, Martignoni M, Galli A. (1990) Finding of a bull with Y;17 traslocation. *Hereditas* 113:63–67.

[12] Patel RK. (1999) A new case of Robertsonian translocation rob (7;16) in HF crossbred bull. *Ind J Dairy Sci* 52:324-329.

[13] Pearce PD, Ansari HA, Maher DW, Malcolm AA, Stewart-Scott IA, Board TE. (1994) New Robertsonian translocation chromosomes in domestic sheep (Ovis aries). Cytogenet. *Cell Genet* 67: 137–140.

[14] Singh B, Fisher KRS, Yadav BR, Basrur PK. (1994) Characterization of a translocation and its impact on fertility in the pig. *Cand J Genome* 37:280–288.

[15] Buoen LC, Zang TQ, Weber AF, Anderson MR, Ruth GR. (1992) The requirement of fibroblasts to confirm the identy of cytogenetic centric fusion (CF) carrier in same-sex twin cattle. *J Vet Diagn Invest* 4: 212–214.

[16] Zhang TQ, Buoen LC, Weber AF, Ruth CR, Anderson ME. (1992) Two different centric fusion chromosomal defects in a Simbrah bull: A case report. *Theriogenology* 37: 553–558.

[17] Rubes J, Musilova P, Borkovec L, Borkovcova Z, Svecava D, Urbanova J. (1996) A new Robertsonian traslocation in cattle, rob (16;20). *Hereditas* 124: 275–279.

[18] Thiagarajan V, Rajasekaran J, Natarajan N. (1990) Robertsonian translocation in cattle, rob (16;20). *Hereditas* 124: 275–279.

[19] Chauhan JB, Patel RK, Singh KM, Soni KJ. (2009) A dicentric Robertsonian translocation, rob (1;29) in Indian Jersey crossbred (Bos taurus x *Bos indicus*) bull. *Nucleus* 52:119–123.

[20] Patel RK, Singh KM, Soni KJ, Chauhan JB. (2006) Novel Cytogenetic finding: an unusual X;X-translocation in Mehsana buffalo (Bubalus bubalis). Cytogent Genom Res 115:186-188.

[21] Vijh RK, Tantia MS, Sahai R. (1994) Translocation in Murrah buffalo. *Ind J Anim Sci* 64:534–538.

[22] Hanada H, Geshi M, Suzuki O. (1995) Additional evidence of formation of unbalanced embryos in cattle with 7/21 Robertsonian translocation. *Theriogenology* 44:499–505.

[23] Hanada H, Geshi M, Sakaguchi M, Yonai M. (1993) Chromosome analyses of embryos sired by bulls heterozygous for the 7/21 robertsonian translocation. *Anim Sci Technol (Jpn)* 64:1178–1183.

[24] Gustavsson I. (1979) Distribution and effect of the 1/29 Robertonian translocation in cattle. *J Dairy Sci* 62:825–855.

[25] Wilson TD. (1991) Monosomy and Trisomy in bovine embryos sired by bulls heterozygous for the 1;29 translocation chromosome. *Theriogenology* 36:789–793.

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RESEARCH ARTICLE OPEN ACCESS

CATALYTIC EFFECTS OF PLANTATION AND MICROBIAL INOCULATION IN NATURAL REGENERATION ON LIMESTONE MINED SPOIL

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ABSTRACT

The experimental plantation of Pongamia pinnata, Jatropha curcas, Ailanthus excelsa and Withania somnifera supplemented with native microbial inoculants produced a catalytic effect on natural regeneration process in planted area on nutrient poor calcareous spoil. The planted area of the spoil showed accelerated immigration of surrounding flora which resulted into enhanced frequency, abundance, relative frequency of pioneering species as compared to unplanted area. Plantation supplemented with microbial inocuation has helped to accelerate jump start succession on mined spoil.

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KEY WORDS

Limestone mine spoils; Plantation; Microbial inoculants; Natural regeneration

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[I] INTRODUCTION

The natural plant succession is the important process in the vegetation development [1]. During mining activities the components of soil ecosystem of affected land is jeopardized. Natural recovery of such a harsh site of un-amended spoil is a slow process as the initial level of many nutrients in the successional process in mine spoil has been in the lower quantity [2, 3]. This is possibly the prime reason for slow vegetational development on un-amended mine spoils. Plantation may alter edaphic conditions and may play as a catalyst for natural regeneration on mine spoils. When the plantation is supplemented with microbial inoculants, Physico-chemical properties of soil are converted suitable for planted species and it allows other species to grow naturally and also provide shade to protect the herbaceous vegetation. Introduction of plant species attracts immigration of naturally grown surrounding plant species and if they established, may result into a distinctive floral cover on mined lands. During ecosystem development on mine spoils, accumulation of nutrients take place and ultimately a self-sustaining ecosystem is developed. Invasion of native herbaceous species along with planted species may play a significant role in increasing the floral diversity [4]. In the present investigation, an effort is made to explain how the plantation supplemented with microbial inoculants stimulates natural regeneration process on completely unvegetated mined out degraded land.

[II] MATERIALS AND METHODS

2.1. Study site

The study area is an important centre of limestone mining activities. There are number of lime stone quarries and lime manufacturing units in operation. Due to extensive mining for lime stone, over burden dumps of mined out waste materials (spoil) is spread all through the area creating heaps or mounds of different age groups. The study site is located at Bistara on Jhukehi-Kaimur road in Katni district of Madhya Pradesh, India. The study site lies between 80° 27'29" E longitude and 23° 58' 36" N latitude at an altitude of 380.0 m (MSL) and the area falls under semi-arid ecological settings

2.2. Plantation and microbial inoculation

An experimental plantation was carried out on completely unvegetated limestone mined out spoil. Prior to planting soil nutrients level were analyzed. The spoil was planted with seedlings of *Jatropha curcas*, *Pongamia pinnata, Withania somnifera* and *Ailanthus excelsa*. All the seedlings were boosted up by inoculation of consortium of microbial inoculants like Arbuscular Mycorrhizal Fungi (AMF), *Pseudomonas fluorescens, Azospirillum sp*. and *Azotobacter sp*. These microbes were native these spoils and were well adapted to such soil conditions. After isolation from spoil, microbes were grown on their respective growth medium and were multiplied in bulk for field application. The seedlings were grown in polyethylene bags filled with soil collected from limestone mined spoils. Soil was ground to suitable size and duly sterilized before raising the seedlings. After sprouting in the seedlings, above consortium of microbial cultures were inoculated and due care was taken to avoid contamination. After six months of inoculation, the seedlings were transplanted to mined spoils and were allowed to acclimatize in

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calcareous soil conditions. At the time of transplantation, again the seedlings were inoculated with consortium of microbial inoculants.

2.3. Planting technique

Before onset of monsoon, pits of 30cm3 were dug at spacing of 2m×2m. There was no soil change made into pits and 6 months old seedlings were used for plantation. The seedlings were gently placed in the pits after removing the polyethylene bags. Seedlings were inoculated with microbial biofertilizers (either singly or in combination) at the time of plantation and the pits were covered with excavated soil immediately. There was sufficient moisture in the soil along the month of peak monsoon (July), which can help the growth and proliferation of inoculated microbial consortium. The application of different treatments was made by putting the inoculum in the rhizosphere of seedlings planted in mine spoil. The broth culture of bacterial inoculum was diluted 4 times and placed in the rhizosphere of plant @30 ml per plant. While AMF culture containing *Glomus mosseae, Glomus intraradices, Glomus deserticola, Gigaspora rosea, Gigaspora margarita, Acaulospora scrobiculata* and *Acaulospora denticulata* were placed in the rhizosphere @50g soil inocula having 300 infective propagules. The mixture of AMF and bacterial inoculant was added @ 50g of AMF inocula mixed with 30 ml. diluted bacterial culture and these were placed in the rhizosphere of the seedlings planted in the limestone mined spoil. The experiment was started in the month of July, 2004 and the observations on natural regeneration were recorded after two years of plantation in the month of August 2006.

[III] RESULTS

Plantation of suitable species supplemented with microbial inoculants has catalyzed the process of invasion of native herbaceous flora on mined spoils. Plantation accelerated the natural succession process. In the present study the pioneering species which occurred in the spoil were *Phyllanthus niruri, Tridax procumbens, Ocimum gratissimum, Argemone mexicana, Zizyphus mauritiana, Acacia nilotica* and *Parthenium hysterophorus* which established through successional process in mined spoil **[Supplementary Table-1]**.In planted area, the natural regeneration of *Phyllanthus niruri* and *Argemone mexicana* was recorded highest with frequency (60% each) and abundance value of 1.8 and 2.2 respectively which was followed by *Tridax procumbens, Ocimum gratissimum, Parthenium hysterophorus, Acacia nilotica* and *Zizyphus mauritiana*. In planted area the abundance value was recorded highest (2.2) for *Argemone mexicana*. In unplanted area, the occurrence of colonizing species was less frequent. *Tridax procumbens* and *Argemone mexicana* represented in higher frequency (40%) followed by *phyllanthus niruri, Ocimum gratissimum, Zizyphus mauritiana Parthenium hysterophorus* and *Acacia nilotica*. The abundance was recorded maximum (1.7) for *phyllanthus niruri* which was followed by *A. mexicana, P. hysterophorus, T. procumbens, O. gratissimum, Zizyphus mauritiana*, and *A. nilotica*. Although each species was occurring in both planted and unplanted area but the frequency of their occurrence and their abundance was greater in planted area as compared to unplanted area of the overburden dump.

[IV] DISCUSSION

This could be the effect of plantation and microbial treatments given to the planted species which promoted the colonizers for establishment. The microbial population in rhizosphere possibly contributed in the availability of nutrients needed by the growing vegetation. In the dolomite mine spoils, plantations of *Gmelina arborea, A. auriculiformis, E. tereticornis* and *P. pinnata* produced the same effects on bauxite mine spoils [5]. Plantations may improve soil through rooting and incorporation of organic matter. With the passage of time there may be some modification in the physico-chemical characteristics in the mined spoil. Improvement of spoil conditions promoted plant succession of mined spoil. It is evident that by plantation, the spoil condition is modified and gradual development of ecosystem took place and resulted into natural succession of herbs and plant species. Thus, it is evident that the plantation on mined spoil initiated the natural succession process. The results of present study are in conformity with the results of other restoration ecologists who observed accelerated rate of natural succession after plantation on mine spoils [6].There are similar reports of higher rate of natural succession after plantation on coal waste dumps [7].

[V] CONCLUSION

Plantation on mined spoil created catalytic effects to restore soil fertility and ameliorate microclimatic conditions. The plantation supplemented with beneficial microbial inoculants has shown greater influence on the natural regeneration process on mined spoil. Consortium of bacterial inoculants and arbuscular mycorrhizal fungi is supposed to accelerate nitrogen fixation and phosphatase enzyme activity in the rhizosphere of plants which would have ensured the supply of nitrogen and phosphorus in the soil. More over, plant cover prevented soil from erosion thus increased the infiltration rate of the water in the soil. All these changes and favorable alteration of soil characteristics caused immigration of surrounding native herb and tree species and resulted into jump start succession on mined site.

REFERENCES

[1] Thompson R, Vogel WG and Taylor DD. [1984] Vegetation and flora of a coal surface-mine in Laurel county, Kentucky. *Castanea* 49: 111–126.

[2] Roberts RD, Marrs RH, Skeffington RA and Bradshaw AD. [1981] Ecosystem development on naturally colonized china clay wastes. I. Vegetation changes and overall accumulation of organic matter and nutrients. *Journal of Ecology* 69: 153–161.

[3] Bradshaw AD and Chadwick MJ. [1980] The restoration of land: the ecology and reclamation of derelict and degraded land, 317pp, Black Well Scientific Publications, Oxford.

[4] Banerjee SK. and Mishra TK. [1999] Ecosystem development on naturally colonized iron mined areas of Madhya Pradesh. *Journal of Tropical Forestry* 15:213–228.
[5] Prasad R and Pandey RK. [1985] Natural plant succession in the rehabilitation of bauxite and coal mine overburden in Madhya Pradesh. *Journal of Tropical Forestry* 1: 79–84.
[6] Richardson AE. [2001] Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Australian Journal of Plant Physiology* 28: 897–906.
[7] Kimber AJ, Pulford ID and Duncan HJ. [1978] Chemical variation and vegetation distribution on a coal waste tip. *Journal of Applied Ecology* 15: 627–633.

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Supplementary Table–1: (As supplied by author)

S. No	Species Appeared	Planted area				Unplanted area			
		Frequency (%)	Relative frequency (%)	Abundance	A/F	Frequency (%)	Relative frequency (%)	Abundance	A/F
1.	*Phyllanthus niruri*	60	19.35	1.8	0.0305	30	15.8	1.7	0.055
2.	*Tridax procumbens*	50	16.12	1.8	0.036	40	15.8	1.5	0.037
3.	*Ocimum gratissimum*	50	16.12	1.3	0.025	30	15.8	1.0	0.033
4.	*Argemone mexicana*	60	19.35	2.2	0.037	40	21.09	1.5	0.037
5.	*Zizyphus mauritiana*	20	6.45	1.0	.05	20	10.5	1.0	0.05
6.	*Acacia nilotica*	30	9.7	1.7	0.057	20	10.5	1.0	0.05
7.	*Parthenium hysterophorus*	40	12.9	1.0	0.025	20	10.5	1.5	0.05

Table 1 -Natural regeneration in planted and unplanted area of limestone mined spoil

RESEARCH ARTICLE OPEN ACCESS

ANTIOXIDANT ACTIVITY OF RAW AND DIFFERENTIALLY PROCESSED UNDER-UTILIZED TROPICAL LEGUME *CANAVALIA ENSIFORMIS* L. DC SEEDS, SOUTH INDIA

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ABSTRACT

In the present study, the antioxidant activity of extracts of raw and differentially processed seed materials of Canavalia ensiformis L.DC (Jack bean) collected from South India was investigated. The raw seeds were found to contain higher levels of phenolic compounds (238.92 mg/100 g seed flour) and flavonoids (68.12 mg/100 g seed flour). By virtue of their hydrogen-donating ability, all the tested extracts were found to exhibit excellent reducing power, with the highest values being recorded in Kellanadukalli accession. Similarly, when compared with synthetic oxidants (Ascorbic acid), all the presently studied jack bean seed extracts were found to be more potent in free radical scavenging activity against α, α - diphenyl - β - picrylhydrazyl (DPPH) radicals. Among the various common processing methods employed in the present study, the cooking treatment was found to improve the antioxidant property of jack bean seed. Hence, such viable and suitable processing method could be recommended for the utilization of such under-utilized legume seeds as a source of natural antioxidants, in addition to their nutrient values.

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KEY WORDS

Jack bean; antioxidant activity; DPPH; phenols; flavonoids

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[I] INTRODUCTION

Most of the free radicals have been implicated in causation of various human ailments such as cancer, rheumatoid arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis and AIDS [1]. Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins [2]. The oxidant radical can be transformed into more reactive forms such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals, which are collectively known as reactive oxygen species (ROS).

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [3]. The Food and Nutrition Board of the National Academy of Science defined a dietary antioxidant as a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species, or both on normal physiological function in humans. In order to prolong the storage of foods, several synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used currently, but these substances may be inappropriate for chronic human consumption, as recent publications have mentioned their possible toxic properties for human health and the environment [4]. Phenolic compounds and antioxidant activities in legume seeds were reported by several earlier communications, although legumes constitute one of the most abundant and least expensive sources of protein in human/animal diet [5]. Phenolic constituents, such as flavonoids, phenolic acids, diterpenes and tannins are especially worthy of notice due to their high antioxidative activity [6]. Natural phenolic antioxidants can scavenge reactive oxygen and nitrogen species (RONS) thereby preventing the onset of oxidative diseases in the body. A positive correlation between the consumption of phenolic-rich foods and a decrease of several chronic diseases has been shown to exist from epidemiological studies [7].

Recently the under-utilized legume seeds have received more attention of the researchers as an alternative protein source all around the world [8]. Sincere and concerted attempts are being made to evaluate and utilize the under-utilized legume seeds as an alternative/additional protein source in the diets of both human beings and animals, particularly developing countries [9]. Among the various under-utilized pulses, the jack bean [Canavalia ensiformis L.DC.] seeds merits a wide use in South Asian countries and other parts of the tropics as food legume. In this connection, the seeds of Canavalia ensiformis L.DC. (Jack bean), an under-utilized food legume becomes more important. C.ensiformis is distributed throughout the tropics and randomly cultivated in South India. The mature seeds of C.ensiformis are being consumed by the Indian tribal sects such as Kurumba,

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Malayali, Irula and other Dravidian groups after cooking [9]. Jack beans are found to contains higher levels of proteins (19.9 – 35.0%), lipids (0.8 – 9.9%) and other nutrients and merits a wide use in South and Southeast Asian countries and other parts of the tropical food legumes [9]. The protein qualities of this wild legume seeds seem to be similar to that of most edible legumes and hence, they are advocated to be a good source of extending protein sources. Several studies revealed that jack bean seeds contain potential of antioxidant activity [10]. Although, few reports are available on the nutritional and antinutritional properties jack bean seeds, the information regarding their antioxidant property was found to be meager. Hence, the present study was carried out with a view to analyze the antioxidant properties of raw and differentially processed seeds samples of jack bean collected from South India. The analysis included 1, 1-diphenyl-picryl-hydrazyl (DPPH), Reducing power assay, quantification of flavonoids and total phenolic compounds (TPC).

[II] MATERIALS AND METHODS

2.1. Collection of the seed samples

Five different accessions of jack bean seed materials were collected from different agroclimatic regions of Western Ghtas, South India from the natural stands [Table–1]. Soon after collection, the immature and damaged seeds were removed and the mature seeds were dried in the sun light for 24 h and stored in plastic containers in refrigerator (50C), until further use.

Table: 1. Collection details of various accessions of *Canavalia sp*.

Location	Seed coat colour	District	State	Date of collection
Bannari	white	Erode	Tamilnadu	22.05.2009
Kuppanatham	Red	Madurai	Tamilnadu	22.05.2009
Ayodhyapattinam	white	Salem	Tamilnadu	29.05.2009
Arachalur	white	Erode	Tamilnadu	29.05.2009
Keelanadukalli	white	The Nilgiris	Tamilnadu	10.06.2009

2.2. Processing methods

Five separate batches of whole seeds of jack beans were taken and the first batch was soaked in distilled water for 24 h at room temperature (30 ± 20 C) in the bean to water ratio of (1:10) w/v. The second batch of seeds was cooked at 90 - 950 C for 1 h in the bean to water ratio of (1:10) w/v. The third batch of seeds were roasted for 30 min at 100 – 110 0 C in an iron pot along with clean fine sand to prevent the burning of the seed coat and to ensure the uniform distribution of heat. The fourth batch of seeds as soaked in distilled water for overnight were germinated in sterile Petri dishes lined with a wet filter paper for 24 h at room temperature (32 ± 20C) in the dark. After each treatment, the treated seeds were rinsed with distilled water, separately, and then dried at 55 0 C for 6 h in a hot air oven. The fifth batch of raw seeds was stored as such without any treatment.

2.3. Preparation of extracts

Seed samples were weighed (10 g each) and blended with Waring blender and soaked with methanol [in ratio seed flour: methanol (1: 10)] for seven days and filtered using Whatman No. 1 paper. The methanol was completely removed by vacuum evaporator at 50oC to give viscous mass. The crude extracts were weighed and stored at 0 – 4oC before analysis.

2.4. DPPH radical scavenging activity

DPPH scavenging activity was carried out by the method of Blois [11]. Two different concentrations (500 & 250 µg/ml) of *Canavalia ensiformis* seed extracts (Methanol) were dissolved in DMSO (dimethyl sulfoxide) and taken in test tubes in triplicates. Then 5 ml of 0.1mM ethanol solution of DPPH (1, 1, Diphenyl-2- Picrylhydrazl) was added to each of the test tubes and were shaken vigorously. They were then allowed to stand at 37oC for 20 minutes. The control was prepared without any extracts. Methanol was used for base line corrections in absorbance (OD) of sample and measured at 517nm. A radical scavenging activity was expressed as 1% scavenging activity and was calculated by the following formula.

$$\text{Radical scavenging activity (\%)} = \frac{\text{OD Control} - \text{OD Sample} \times 100}{\text{OD control}}$$

2.5. Reducing power assay

Reducing activity was carried out by using the method of Oyaizu [12]. Two different concentrations (500 & 250 µg/ml) of Canavalia ensiformis seed extracts (Methanol) were dissolved in DMSO (dimethyl sulfoxide) and taken in test tubes in triplicates. To the test tubes, 2.5 ml of sodium phosphate buffer and 2.5 ml of 1% Potassium ferric cyanide solution was added. These contents were mixed well and were incubated at 50oC for 20 minutes. After incubation 2.5ml of 10% TCA was added and were kept for centrifugation at 3000rpm for 10 minutes. After centrifugation 5ml of supernatant were taken and to this 5ml of distilled water was added. To this about 1ml of 1% ferric chloride was added and was incubated at 35o C for 20 minutes. The O.D (absorbance) was taken at 700nm and the blank was prepared by adding every other solution but without extract and ferric chloride (0.1%) and the control was prepared by adding all other solution but without extract.

2.6. Total phenols

Total phenolic contents were determined by McDonald et al. [13] method. A dilute extract of each seed extract (0.5 ml of 1:10g ml –l) or garlic acid (standard phenolic compound) was mixed with 5 ml of Folin Ciocalteu reagent (1:10 diluted with distilled water) and 4 ml of aqueous sodium carbonate (1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by measuring absorbance at 765

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nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg/ml solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g-l of dry mass), which is a common reference compound.

2.7. Flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination [14]. Each seed extracts (0.5ml of 1:10 g/ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). The calibration curve was prepared by preparing quercetin solution at concentrations 12.5 to 100g ml -1 in methanol.

[III] RESULTS

In the present study, the seed materials of five different accessions of an under-utilized legume, Jack bean were collected from different agro-ecological regions of South India. After collection, the antioxidant (DPPH and reducing power assay) properties and the levels of poly phenols and flavonoids were analyzed and the results were expressed in the **Tables 1-7**.

3.1. DPPH assay

Radical-scavenging activity employing DPPH has been extensively used in the field of food processing for screening the antioxidant capacity of agricultural produce [15] (Sanchez-Moreno, 2002). The assay was carried out in the methanol extracts with two different concentrations like 500 and 250µg/ml. The antioxidant activity of raw seeds of five different jack bean accessions was found to be ranged from 38.04 – 68.51%. Highest antioxidant activity was recorded by Arachalur accession **[Tables– 2 and 3]**.

3.2. Reducing power assay

The reducing power assay of five different accessions of raw jack bean seeds were given in table 4 and 5, which was found to be ranged from 0.202-0.722 of the seed flour. Among the five different accessions, the Ayodhyapattinam germplasm have exhibited the highest reducing activity.

3.2. Total phenols and flavonoids

The total phenols content of five different accessions of raw jack bean seed flour was found between 135.47 and 238.92 mg/100g of seed flour (Table 6). Among the five different accessions, the Keelanadukalli germplasm showed the highest levels of poly phenols. The flavonoids content of five different accessions of raw jack bean seed flour was found between 52.05 - 68.12 mg/100g of seed flour (Table 7). Bannari germplasm has registered the highest level of flavonoids.

Table: 2. Antioxidant activity (DPPH assay) of raw and moist heat treated jack bean (*Canavalia ensiformis*)*

Accessions	Percentage of Free Radical Scavenging Activity					
	Raw Seeds (µg/ml)		Soaking (µg/ml)		Cooking (µg/ml)	
	250	500	250	500	250	500
Bannari	45.61 ± 0.603	56.42 ± 0.419	30.91 ± 0.135	53.79 ± 0.123	28.72 ± 0.190	72.45 ± 0.045
Ayodhyapattinam	38.04 ± 0.047	63.6 ± 0.230	28.47 ± 0.095	50.86 ± 0.112	34.79 ± 0.202	73.47 ± 0.170
Arachalur	42.61 ± 0.213	68.51 ± 0.215	22.28 ± 0.05	52.63 ± 0.088	40.45 ± 0.220	75.17 ± 0.090
Kuppanatham	45.42 ± 0.14	57.6 ± 0.075	28.24 ± 0.210	48.54 ± 0.205	39.24 ± 0.100	74.47 ± 0.182
Keelanadukalli	42.42 ± 0.120	66.35 ± 0.183	29.51 ± 0.181	50.13 ± 0.280	34.85 ± 0.150	75.88 ± 0.040

Table: 3. Antioxidant activity (DPPH assay) of raw, germination and dry heat treated jack bean (*Canavalia ensiformis*)*

Accessions	Percentage of Free Radical Scavenging Activity					
	Raw Seeds (µg/ml)		Roasting (µg/ml)		Germination (µg/ml)	
	250	500	250	500	250	500
Bannari	45.61 ± 0.603	56.42 ± 0.419	33.25 ± 0.060	66.14 ± 0.083	42.39 ± 0.240	70.83 ± 0.005
Ayodhyapattinam	38.04 ± 0.047	63.6 ± 0.230	36.60 ± 0.133	65.81 ± 0.046	39.61 ± 0.035	74.76 ± 0.057
Arachalur	42.61 ± 0.213	68.51 ± 0.215	35.13 ± 0.066	69.52 ± 0.080	36.48 ± 0.036	74.39 ± 0.147
Kuppanatham	45.42 ± 0.14	57.6 ± 0.075	29.33 ± 0.068	66.57 ± 0.109	32.91 ± 0.062	72.8 ± 0.1
Keelanadukalli	42.42 ± 0.120	66.35 ± 0.183	28.43 ± 0.085	68.27 ± 0.280	32.42 ± 0.098	70.66 ± 0.149

*The data are means of triplicate determinations and ± standard errors

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Table: 4. Antioxidant activity (reducing power assay) of raw and moist heat treated jack bean (*Canavalia ensiformis*)*

Accessions	Raw Seeds (µg/ml)		Soaking (µg/ml)		Cooking (µg/ml)	
	250	500	250	500	250	500
Bannari	0.336 ± 0.002	0.686 ± 0.001	0.340± 0.002	0.549 ± 0.001	0.347 ± 0.00	0.763 ± 0.001
Ayodhyapattinam	0.327 ± 0.002	0.722 ± 0.001	0.227 ±0.001	0.610 ± 0.001	0.333 ± 0.001	0.790 ± 0.001
Arachalur	0.221 ± 0.002	0.488 ± 0.002	0.243 ± 0.001	0.523 ± 0.002	0.318 ± 0.003	0.791 ± 0.009
Kuppanatham	0.247 ± 0.001	0.685 ± 0.003	0.331 ± 0.001	0.563 ± 0.002	0.297 ± 0.00	0.828 ± 0.002
Keelanadukalli	0.202 ± 0.002	0.430 ± 0.00	0.333 ± 0.001	0.578 ± 0.002	0.302 ± 0.001	0.840 ± 0.001

*The data are means of triplicate determinations and ± standard errors

Table: 5. Antioxidant activity (reducing power assay) of raw, germination and dry heat treated jack bean (*Canavalia ensiformis*)*

Accessions	Raw Seeds (µg/ml)		Roasting (µg/ml)		Germination (µg/ml)	
	250	500	250	500	250	500
Bannari	0.336 ± 0.002	0.686 ± 0.001	0.395 ± 0.001	0.720 ± 0.001	0.295 ± 0.002	0.720 ± 0.00
Ayodhyapattinam	0.327 ± 0.002	0.722 ± 0.001	0.296 ± 0.001	0.759 ± 0.001	0.353 ± 0.003	0.793 ± 0.001
Arachalur	0.221 ± 0.002	0.488 ± 0.002	0.263 ± 0.002	0.682 ± 0.010	0.301 ± 0.001	0.795 ± 0.001
Kuppanatham	0.247 ± 0.001	0.685 ± 0.003	0.455 ± 0.003	0.751 ± 0.003	0.408 ± 0.001	0.824 ± 0.00
Keelanadukalli	0.202 ± 0.002	0.430 ± 0.00	0.351 ± 0.009	0.422 ± 0.002	0.421 ± 0.001	0.750 ± 0.002

*The data are means of triplicate determinations and ± standard errors

Table: 6. Quantitative estimation of Poly phenols in raw and differentially processed seed samples of *Canavalia ensiformis**

Accessions	Poly phenols content (mg/100 g seed flour)				
	Raw Seeds	Soaking	Cooking	Roasting	Germination
Bannari	146.58 ± 8.22	113 ± 0.25	121 ± 0.05	130.4 ± 0.02	142 ± 0.24
Ayodhyapattinam	152.45 ± 0.04	118.1 ± 0.24	128 ± 0.18	132.7 ± 0.35	137 ± 0.25
Arachalur	135.47 ± 2.16	108 ± 0.04	106.2 ± 0.06	125.5 ± 0.12	127.28 ± 0.06
Kuppanatham	221.29 ± 0.03	118.39 ± 0.05	113.84 ± 0.05	138.69 ± 0.23	184 ± 0.08
Keelanadukalli	238.92 ± 0.67	179.2 ± 0.18	132.66 ± 0.05	124.24 ± 0.14	121.81 ± 0.13

*The data are means of triplicate determinations and ± standard errors

Table: 7. Quantitative estimation of Flavonoids in raw and differentially processed seed samples of *Canavalia ensiformis*

Accessions	Flavonoids content (mg/100 g seed flour)				
	Raw Seeds	Soaking	Cooking	Roasting	Germination
Bannari	68.12 ± 0.25	28.93 ± 0.07	43 ± 0.01	32.25 ± 0.74	32.80 ± 0.02
Ayodhyapattinam	54.25 ± 0.24	30.77 ± 1.02	30.21 ± 1.71	35.65 ± 2.05	40.14 ± 0.31
Arachalur	52.05 ± 0.46	37.7 ± 0.14	31.62 ± 0.42	28.52 ± 1.33	37.46 ± 0.18
Kuppanatham	59.92 ± 0.11	38.80 ± 0.32	34.60 ± 1.59	36.14 ± 0.29	42.1 ± 0.31
Keelanadukalli	61.28 ± 0.24	42.45 ± 0.31	39 ± 0.28	27.08 ± 4.21	46.4 ± 0.05

*The data are means of triplicate determinations and ± standard errors

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[IV] DISCUSSION

The α, α,diphenyl-β-picrylhydrazyl (DPPH) a stable nitrogen centered free radical, has been used to evaluate the antioxidant activity of natural products by measuring the radical quenching capacity in a relatively short period of time. All the presently studied seed extracts (both raw and differentially processed seeds) were found to exhibit more effective free radical inhibition activity against DPPH. The free radical inhibition activity of raw seed materials of jack bean was ranged between 38.04 and 68.51%, which is in agreement with that of the previous reports on *Phaseolus vulgaris* [16]; *Vigna aconitifolia* [17]; *Mucuna pruriens var.utilis* [18]. Among the five different accessions, the arachalur germplasm showed the highest free radical scavenging activity.

When considering the effect of various common processing methods on the free radical inhibition activity of seed extract in the presently studied wild legume grain, all the processed samples showed moderate to higher levels of free radical inhibition activity than those of raw seeds **[Tables–2 and –3]**. The free radical inhibition activity of seed extracts of cooked samples were found to be higher (72.45, 73.47, 75.17, 74.47 and 75.88%) than that of other processed seed samples of the present study and previous reports of free radical inhibition activity of processed samples of Arachis hypogea (23.69%) [2].

Reducing power assay is often to evaluate the ability of natural antioxidant to donate electron or hydrogen [19]. Samples with high reducing power were reported to have a better ability to electrons. It has been widely accepted that the higher level of absorbance at 700 nm indicates greater reducing power of the test samples [20]. Two different concentrations (500 & 250µg/ml) of the seed extracts of raw jack bean seeds were found to When considereing the effect of various common processing methods on the reducing power of jack bean seeds of the present study, all the processed samples showed higher level of reducing power than those of raw seeds **[Tables–6 and –7]**. However, among the differentially processed samples, the extracts of cooked samples registered the maximum level of reducing power in similar with earlier report in Arachis hypogeal [2].

Phenolic and other phytochemical antioxidants found in fruits, vegetables and legumes are bioactive compounds capable of neutralizing free radicals and may play a role in the prevention of certain diseases. Also, dietary supplements and food fortification may be an alternative route to the consumption of minor plant components that may have health effects. A significant level of polyphenols and flavonoids was reduced during soaking treatment in jack bean seeds **[Table–7]**.The level of elimination of phenolic compounds in the presently studied seed materials was found to be higher when compared to a previous report on cowpea and pigeonpea [21]. Since the phenolic compounds are water soluble in nature and mostly located in the seed coat, the decrease on the level of phenolic compounds during soaking treatment might be due to leaching out into the soaking medium.

Effect of various simple and cost-effective processing methods on the levels of antioxidant properties of five different accessions of jack bean seeds were given in tables 2-5. Among the various post-harvest treatments employed, cooking was showed the highest antioxidant activity (72.45 – 75.88%). The effect of various simple and cost-effective processing methods on the levels of phenolic constituents of five different accessions of jack bean seed was presented in **tables –6 and –7**. Among the different processing methods employed in the present study cooking was showed the higher levels of flavonoids and poly phenols (43 – 46.4 mg/100g of seed flour & 132 - 184 mg/100g of seed flour).

[V] CONCLUSION

The results of the present study demonstrated that the seed materials of jack bean constitute a rich source of antioxidants and other secondary metabolites in addition to appreciable levels of various bioactive compounds. All the bioactive compounds in jack bean grains were found to exhibit potential antioxidant activity through in vitro model. When considering the effect of various common processing methods on the antioxidant property of jack bean seeds, the cooking appear to be more effective. Hence such a viable processing method could be recommended for the versatile utilization of jack bean seed as source of natural antioxidants, in addition to protein and other nutrients.

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[1] Pourmorad F, Hosseinimehr SJ, Shahabimajd N. [2006] Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *Afr J Biotech* 5:1142–1145.
[2] Lee KG, Mitchell AE, Shibamoto T. [2000] Determination of antioxidant properties of aroma extracts from various beans. *J Agri. Food Chem*. 48: 4917–4820.
[3] Klein BP, Kurilich AC. [2000] Processing effects on dietary antioxidants from plant foods. *HortScience* 35: 580–584.
[4] Nan Wu, Kuang Fu, Yu-Jie Fu, Yuan-Gang Zu, Fang-Rong Chang, et al. [2009] Antioxidant Activities of Extracts and Main Components of Pigeonpea [*Cajanus cajan* (L.) Millsp.] Leaves. *Molecules* 14: 1032–1043.
[5] Kandhasamy Sowndharajan, Perumal Siddhuraju and Sellamuthu Manian. [2010] In-vitro evaluation of the antioxidant activities in the differentially processed seeds from underutilized legume, Bauhinia vahilii Wight and *Arn. Food Sci Biotechnol* 19(2): 503–509.
[6] Radoslaw Pilarski, Henryk Zielinski, Danuta Ciesiolka, Krzysztof Gulewicz. [2006] Antioxidant activity of ethanolic

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ISSN: 0976-3104

and aqueous extracts of *Uncaria tomentosa* (Willd.) DC. *J Ethnopharmacol* 104: 18–23.

[7] Ryszard Amarowicz, Isabell Estrella, Teresa Hernandez, Montserrat Duenas, Agnieszka Troszynska, et al. [2009] Antioxidant Activity of a Red Lentil Extract and Its Fractions. *Int J Mole Sci* 10: 5513-5527.

[8] Vadivel V, Pugalenthi M. [2007] Biological value and protein quality of raw and processed seeds of Mucuna pruriens var. utilis. *Livestock Research for Rural Development* 19.

[9] Janardhanan K, Vadivel V, Pugalenthi M. [2003] Biodiversity in Indian under-exploited / tribal pulses. In: Improvement strategies for Leguminosae Biotechnology (editors: P K Jaiwal and R P Singh), pp. 353–405. Kluwer Academic Publishers, Brita.

[10] David Betancur-Ancona, Gwendolyna Peraza-Mercado, Yolanda Moguel-Ordonez, Sonia Fuertes-Blanco. [2004] Physicochemical characterization of lima bean (Phaseolus lunatus) and jack bean (*Canavalia ensiformis*) fibrous residues. *Food Chemistry* 84: 287–295.

[11] Blois MS. [1958] Antioxidant determinations by the use of stable free radical. *Nature* 81: 1199–2000.

[12] Oyaizu M. [1986] Studies on products of browning reaction: Antioxidant activities of products of browning reaction prepared from glucosamine. *Jap J Nut* 44: 307–315.

[13] McDonald S, Prenzler PD, Autolovich M, Robards K . [2001] Phenolic content and antioxidant activity of olive extracts. *Food Chemistry* 73: 73–84.

[14] Chang C, Yang M, Wen H, Chern J. [2002] Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug and Anal* 10: 178–182.

[15] Sanchez-Moreno C. [2002] Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci Tech Int* 8: 121–137.

[16] Dave Oomah B, Cardador-Martinez A, Loarca-Pina G. [2005]Phenolics and antioxidant activities in common beans (*Phaseolus vulgaris* L.). *J Sci Food and Agri* 85: 935–942.

[17] Siddhuraju P. [2006] The antioxidant activity and free radical scavenging capacity of phenolics of raw and dry heated moth bean (*Vigna aconitifolia Jacq. Marechal*) seed extracts. *Food Chemistry* 99:149–157.

[18] Siddhuraju and Becker [2003]. Studies on antioxidant activities of Mucuna seed (*Mucuna pruriens var.utilis*) extract and various non-protein amino/imino acids through in-vitro models. *J Sci Food Agri* 83: 1517–1524.

[19] Dorman HM, Kosar K, Kahlos Y, Holm, Hiltunen R. [2003] Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. *J Agri and Food Chem* 51: 4563–4569.

[20] Duh PD, Yen WJ, Du PC, Yen GC. [1977] Antioxidant activity of Mung bean hulls. *J Amer Oil Chem Soc* 74:1059–1063.

[21] Onwuka GI. [2006]Soaking, boiling and antinutritional factors in pigeonpea (Cajanus cajan) and cowpea (*Vigna unguiculata*). *J Food Proc.Preser* 30: 616–630.