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Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4th edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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Biochemical profile of five species of cyanobacteria isolated from polythene surface in domestic sewage water of Silchar town, Assam (India)

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Abstract

Disposal of polythene into waste water poses a serious problem as they get accumulated in the environment. Submerged polythene in waste water offers an ideal substratum for algae to colonize. The present paper highlights the biochemical composition of five cyanobacteria isolated from submerged polythene surface in domestic sewage water, Silchar town, Assam, (India). The carbohydrate, protein, lipid, vitamin C and pigments (Chl*a*, carotenoids, phycobiliproteins) contents of five cyanobacterial species, *Phormidium lucidum*, *Oscillatoria subbrevis*, *Lyngbya diguetii*, *Nostoc carneum*, and *Cylindrospermum muscicola* isolated from submerged polythene surface were analysed. Maximum amount of total protein, carbohydrate and lipid content were found in *Oscillatoria subbrevis* and minimum in *Cylindrospermum muscicol*a. Vitamin C was found to be highest in *Oscillatoria subbrevis* and *Nostoc carneum* and minimum in *Cylindrospermum muscicola*. The total phycobiliproteins was maximum in *Oscillatoria subbrevis* and minimum in *Cylindrospermum muscicola*.One-way analysis of variance (ANOVA) showed significant differences among the biochemical parameters of cyanobacteria isolated from polythene surface.

Keywords: biochemical; cyanobacteria; domestic sewage water; polythene bags; Assam

Introduction

Cyanobacteria are known to occupy a broad range of habitats across all latitudes and are believed to be the earliest inhabitants of earth. They are not only widespread in freshwater, marine and terrestrial ecosystems but also occur in extreme habitats such as hot springs, hypersaline localities, freezing environments and arid deserts (1). Besides such natural habitats, algae including cyanobacteria are capable of growing on artificial substrates as well. Made from non-renewable fossil fuel, introduced around 1970s(2), plastic carry bags are indiscriminately dumped into landfills worldwide and emit dangerous methane and carbon dioxide gases during their decomposing stages as well as highly toxic leachates (3). It effectively blocks sewerage pipe lines, litters agricultural lands, canals, rivers and oceans. They are not biodegradable or take incredibly long time to break down into powdery plastic dusts which contaminate the soil and the water adversely affecting all life forms (4). Algae are known to colonise on polythene surfaces submerged in waste water(5-6).Study of growth of algal species on such polythene substrata are important in the context of biodegradation of polythene (7). Biodegradation of polyethylene by algae constitute an attractive environment friendly and cost effective viable option(8).Algae and cyanobacteria are rich source of several bioactive compounds such as

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proteins, polyunsaturated fatty acids (PUFAs), sterols,enzymes,vitamins and pigments(9).Their vast potential in varied applications such as food,feed, fuel, fertilizer, medicine,industry and in combating pollution(10-16) have been explored. Given the huge diversity of algae and the phytochemicals they produce, exploring their biochemical contents has remained a favourite pastime of researchers.Accordingly, the present study addresses the biochemical screening of five cyanobacteria isolated from submerged polythene surface in the domestic sewage water of Silchar town in the state of Assam, India.

Material and Method

Study area : The study was carried out in the urban area of Silchar town of Cachar district located in the state of Assam, India (Fig. 1) during the 2014. The study area lies between latitude $24^0$49Ê North and longitude $92^0$48$^{\hat{E}}$ East and altitude of 114.69 meters above sea level on the banks of river Barak. The domestic sewage drains carries waste from household and medium scale industries. A view of the study site showing algae colonized on polythene bags is presented in Fig. 2

Physico-chemical properties of sewage water : The water samples from domestic sewage drains were collected, transferred into pre-cleaned plastic bottles and stored for further analysis. The pH was measured using a digital pH meter. Biological oxygen demand (BOD) and dissolved oxygen (DO) measured by titrimetric method (17). Chemical oxygen demand (COD) was measured by open reflux method. Alkalinity, free CO_2 and magnesium and calcium were measured by titrimetric method (18). Total dissolved solid (TDS) and suspended solid (SS) were measured by gravimetric method. Chloride was measured by argentometric method (19). Sulphate was estimated by turbidimetric method (17). Nitrate was measured by brucine method (20). Soluble reactive phosphate was estimated by molybdate blue method(21). Ammonia was determined by phenol-hypochlorite method (22).

Isolation of cyanobacteria : A total of 20 dumped waste polythene bags colonised by algae were collected from domestic sewage water drains of Silchar town, Assam and brought into laboratory.

Fig. 1. Map of the study area showing the location of study sites

Fig.2. Close view of algae colonizing on submerged polythene bags (2A-2B)

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The polythene bags were cut into 1cm^2 size pieces with a sterilized blade. The algae samples from polythene surfaces were scrubbed with a sterilized brush and observed under microscope. The method used for isolation and purification of cyanobacteria was according to Rippka *et al*. (23). The algal samples were homogenized in sterile water with glass beads, centrifuged at 3000 rpm for 10 minutes with repeated washing.The pellets were suspended in sterilized BG-11 medium and placed onto the agar petri plates by pour plate method. The plates were incubated for 15 days under continuous illumination (2000lux) at 24±1^{0}C. The pure colonies developed in the agar plates were picked upandsub-cultured in 500ml Erlenmeyer flasks. The cultures were observed under microscope and the isolated cyanobacterial species were identified using standard keys (24-25). Photomicrographs of five cyanobacterial species were presented in Fig.3.The BG-11 medium without combined nitrogen source was used for the isolation and maintenance of *Nostoc*and *Cylindrospermum.*

Biochemical analysis : The total carbohydrate was determined according to anthrone method (26). Total protein was estimated by modified method of Herbert*et al*.(27).The chl*a* and carotenoids were estimated by the standard methods of Strickland and Parsons (28) and Parson (29), respectively. Phycobiliproteins estimation has been carried out as per Bennet and Bogorad (30). Lipid content was estimated by the standard method of Bligh and Dyer (31). Vitamin C content was evaluated using the method of Roe and Keuther (32). Growth rate was measured in terms of chl *a* as biomass component(33). Growth kinetics in terms of specific growth rate (K) and generation time(G) were evaluated (34).

Statistical analysis : The statistical analyses were performed using the software Statistical Package for Social Sciences (SPSS Version 21.0). One-way analysis of variance (ANOVA) was used to

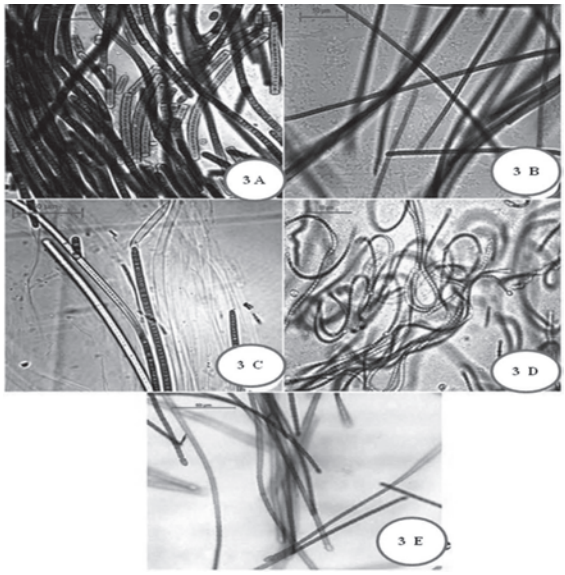

Fig. 3. Photomicrographs of five cyanobacteria isolates
3 A- Phormidium lucidum, 3 B- Oscillatoria subbrevis,
3 C- Lyngbya diguetii, 3D- Nostoc carneum,
3 E- Cylindrospermum muscicola

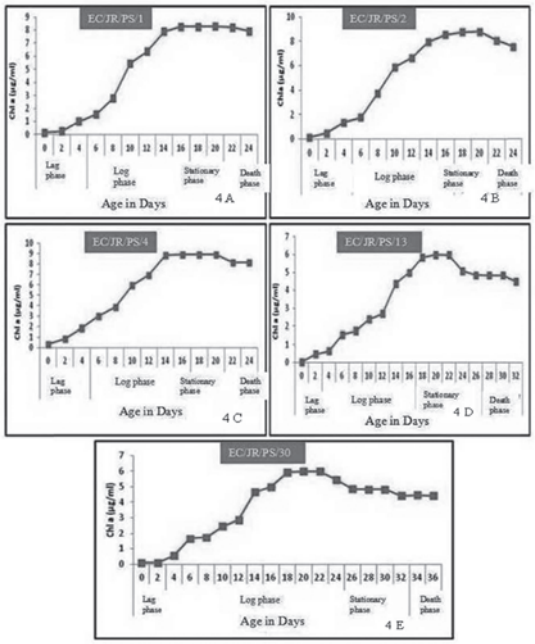

Fig. 4. Growth curve of isolated cyanobacteria (4 A-Phormidium lucidum, 4B- Oscillatoria subbrevis , 3 4C- Lyngbya diguetii, 4D- Nostoc carneum, 4 E-Cylindrospermum muscicola)

evaluate the differences among the biochemical parameters.The triplicate sets of data were evaluated inaccordance with the experimental design (Completely Randomized Design) with ANOVA (Analysis of Variance). The comparisons between the different means were made usingpost hoc least significant differences (LSD) calculated at p level of 0.05 (5%), and representedas CD (Critical Differences) values in Table 3 and 4 with standard deviations.

Results

The physico chemical propertites of domestic sewage water are presented in Table 1. The colour of domestic sewage water was black to yellowish grey. The temperature ranged from 28 to 34°C. The site 2 recorded maximum water temperature while site 4 recorded the minimum. The pH values of the different sites were quite at variance with each other. The domestic sewage water of site 2 was slightly acidic while that for site 4 was found to be alkaline.The value of BOD varied from 383 to 600 mg/L with site 3 registering maximum value. The COD values were in the range of 1511 to 2189 mg/L with maximum being at site 5. The DO concentrations varied from 1.3 to 2.4mg/Lwith maximum being at site 4 and minimum at site 1. Alkanity of domestic sewage water varied from 9 to 11mg/L. Free CO_2 was in the range 38-42mg/L with maximum at site 2. Nitrate ranged from 12 to 65 mg/L with maximum being at site 4. Magnesium ranged from 25 to 178mg/L, maximum being at site 4 and minimum at site 2. The TDS of sewage water were within the range of 500 to 3210mg/L, it was maximum being at site 2 .The SS of sewage water was in the range of 50 to 200mg/L and being it was maximum at site 2 and minimum at site 3. The chloride concentration were in the range of 35 to 78mg/L, being maximum at site 2 .The calcium content of sewage water was found in the range 54 - 69mg/L, , being maximum at site 4. The sulphate of domestic sewage varied from a minimum of 50mg/L at site 3 to maximum of 897mg/L at site 4.The minimum ammonia value was found to be 28mg/L at site 1 andmaximum at34mg/L at site 4.The phosphate of sewage water varied from a minimum of 58mg/L at site 5 to a maximum of 72mg/L at site 4.

The growth curves of five cyanobacterial species were presented in Fig.4. The maximum growth rate (Table 2)has been shown by *Oscillatoria subbrevis*(0.158μd^{-1})followed by *Nostoc carneum* (0.152μd^{-1}). The growth rate was lowest in *Phormidium lucidum* (0.134μd^{-1}).The generation time was maximum in *Phormidium lucidum* (178.25h) and minimum in *Oscillatoria subbrevis* (151.34h).

The biochemical analysis of five species of cyanobacteria (Table 3) revealed the carbohydrate to be in the range109-370 μgml^{-1}**.**The maximum carbohydrate present in *Oscillatoria subbrevis* (370 μgml^{-1}**)** and minimum in *Cylindrospermum muscicola* (109 μgml^{-1}**)**.The carbohydrate present in *Phormidium lucidum*, *Lyngbya diguetii*, *Nostoc carneum* were 240 μgml^{-1}**,** 230 μgml^{-1} and 113 μgml^{-1}, respectively**.** The protein range was145-230 μgml^{-1}.The maximum protein content was found in *Oscillatoria subbrevis* (230 μgml^{-1}**)** and minimum in *Cylindrospermum muscicola* (145 μgml^{-1}**)**. The protein present in *Phormidium lucidum*, *Lyngbya diguetii*, *Nostoc carneum* were 210 μgml^{-1}, 203 μgml^{-1} and 195 μgml^{-1}. The range of vitaminC was 0.3-0.9 μgml^{-1}. The maximum vitamin C content was observed in *Oscillatoria subbrevis*and *Nostoc carneum*(4.2μgml^{-1}) , minimum in *Cylindrospermum muscicola* (1.2 μgml^{-1}). The vitaminC present in *Phormidium lucidum*, *Lyngbya diguetii* were 0.5 μgml^{-1} and 0.8 μgml^{-1},respectively.The range of lipid content in cyanobacterial isolates was 4.2-11.2μgml^{-1}. The maximum lipidcontent was noted in *Oscillatoria subbrevis* (11.2 μgml^{-1}) and minimum in *Cylindrospermum muscicola* (4.2 μgml^{-1}**)**. The lipid content present in *Phormidium lucidum*, *Lyngbya diguetii*, *Nostoc carneum* were 8.7 μgml^{-1},7.3 μgml^{-1} and 5.1 μgml^{-1} ,respectively.

Phycocyanin (PC) content (Table 4) was maximum in *Lyngbya diguetii* (17.5 μgml^{-1}) and minimum in *Nostoc carneum* (12.1 μgml^{-1}). Phycoerythrin (PE) content was maximum in *Oscillatoria subbrevis* (48.7 μgml^{-1}**)** and minimum

in *Cylindrospermum muscicola* (15.34 μgml^{-1}). Allophycocyanin (APC) was maximum in *Lyngbya diguetii* (25 μgml^{-1}**)** and minimum in *Cylindrospermum muscicola* (15.34 μgml^{-1}**)**. Total phycobiliproteins content was maximum in *Oscillatoria subbrevis* (81.4 μgml^{-1}) and minimum in *Cylindrospermum muscicola* (45.98 μgml^{-1}**)**.

One way ANOVA revealed significant differences among the biochemical parameters, chl*a* ($p = 0.02$), carotenoids ($p = 0.01$), protein ($p = 0.02$), carbohydrates ($p = 0.04$), vitaminC ($p = 0.02$), lipids ($p = 0.04$). Significant variationin phycobiliproteins concentrations, PE ($p = 0.02$), PC ($p = 0.01$) and APC ($p = 0.04$) were observed.It is noteworthy that total phycobili proteins in *Oscillatoria subbrevis* contains almost double the amount of *Cylindrospermum muscicola* **.**

Discussion

The biochemical constituents of cyanobacteria isolated from polythene surface submerged in domestic sewage water showed that *Oscillatoria subbrevis*, *Phormidium lucidum*, *Lyngbya diguetii*, *Nostoc carneum* and *Cylindrospermum muscicola* contain high cellular constituents of chl*a*, carotenoids, protein, carbohydrate, vitamin C, lipid and phycobili proteins. Significant differences were observed in biochemical constituents species wise. In the present study, *Oscillatoria subbrevis* was found to thrive in an alkaline condition (pH = 8.1, site 4) while *Phormidium lucidum* was collected from an acidic (pH = 5.8, site 2) sewage water. The species *Lyngbya diguetii isolated* was found to grow under slightly acidic sewage water (pH = 6.3, site 3) and *Nostoc carneum* and *Cylindrospermum muscicola* was collected from slightly alkaline condition (pH = 7.3, site 1) and moderately acidic condition (pH = 6.4**,** site 5)**,** respectively.The characteristic morphological and physiological attributes of the species might be ascribed to typical physico-chemical properties of domestic sewage water. It has been reported that the cellular composition of cyanobacteria depend on the nature of strains, physiological state of the isolates and the nutrient conditions of environment from where they have collected (35-38).

The dissolved oxygen of domestic sewage water varied from 1.3-2.4mg/L. The growth of algae was found to be directly proportional to the available nutrients and oxygen level of water which in turnmight alter the oxygen level of sewage water.The cyanobacteria in the present study has been found to adapt well to the oxygen depleted condition of the sewage water and it was presumed that in the absence of additional nitrogen source in domestic sewage water, the flow of carbon fixed in photosynthesis is switched from the path of protein synthesis to that leading to higher production of biochemical constituents of microalgae (39).The extent of dissolved solid and suspended solid of domestic sewage water was quite different in all the five sites. Therefore, the sunlight penetration on submerged polythenes is anticipated to be different.Grossman *et al*.(40) opined that environmental condition of species might alter the composition and abundance of phycobiliproteins. In the present study, the physico chemical parameters were at variance in all the five sites. This, we believe, might have caused a variation in the total phycobiliproteins in the species studied.

In a previous study, the algal species *Phormidium angustissimum*, *Lyngbya holdenii*, *Anabaena doliolum*, *Calothrix marchica* and *Fischerella muscicola* isolated from lime sludge waste of a paper mill in the district showed higher accumulation of chl *a*, phycocyanin, carbohydrates and protein (41). Lime sludge waste is rich in organic carbon and thus contributes to the nutrition of cyanobacterial growth. Lipids content was recorded highest in *Oscillatoria subbrevis* and lowest in *Cylindrospermum muscicola*.In the present study, carbohydrate and protein are found to be highest in *Oscillatoria subbrevis* and lowest in *Cylindrospermum muscicola*, respectively. This is in conformity with the observation made by Zhu *et al.* (42) wherein it has been shown that proteins are present as large fraction of biomass in growing algae.

Table 1: Physico-chemical properties of domestic sewage drain water

Water Parameters	Site 1 (Link road)	Site 2 (Sonai road)	Site 3 (NH road)	Site 4 (Premtola)	Site 5 (Ambikapatty)
Colour and odour	*Black, present*	*Black, present*	*Black, present*	Yellowish grey, present	Yellowish grey, *present*
Temperature	32°C	34°C	29°C	28°C	35°C
pH	7.3±0.23	5.8±0.10	6.3±0.13	8.1±0.23	6.4±0.21
BOD (mg/l)	586.3±0.45	483±0.14	600±0.18	383±1.2	509±2.3
COD (mg/l)	1511±0.67	1520±0.56	1520±0.18	1764±0.24	2189±0.78
DO (mg/l)	1.3±0.12	2.3±0.15	2.2±0.23	2.4±0.21	2.2±0.12
Alkalinity(mg/l)	9±0.34	9.8±0.12	10±1.4	11±0.12	9.8±0.23
Free CO_2 (mg/l)	38±0.13	42±0.13	36.98±0.13	39±0.21	36±1.2
TDS (mg/l)	500±1.2	3210±1.4	500±0.14	1546±2.4	578±2.5
Suspended solids (mg/l)	51±0.56	200±0.13	50±0.35	53±0.13	58±0.23
Chlorides(mg/l)	62±0.21	78±0.34	60±0.06	73±0.21	35±0.12
SO_4^{-2} (mg/l)	880±1.3	876±1.4	50±1.6	897±3.2	783±0.23
Nitrate (mg/l)	43±0.13	44±0.12	12±1.5	65±0.13	46±1.2
Mg (mg/l)	32±0.24	25±0.67	30±1.1	178±1.3	176±2.1
Ammonia (mg/l)	28±0.12	32±0.34	30±1.2	34±0.12	32±0.23
Appearance	Not clear	Not clear	Not clear	Not clear	Not clear

Table 2: Specific growth rate (K) and generation time (G) of the isolates

Sl. No	Cyanobacterial isolates	K (μd^{-1})	G (h)
1	*Phormidium lucidum*	0.134	**178.25**
2	*Oscillatoria subbrevis*	**0.158**	151.34
3	*Lyngbya diguetii*	0.138	173.67
4	*Nostoc carneum*	0.152	157.21
5	*Cylindrospermum muscicola*	0.136	153.89

Vitamin C, a wide spectrum antioxidant not synthesized in the body is obtained from dietary sources (43). In the present study, vitamin C contents was found to be highest in *Oscillatoria subbrevis*, *Nostoc carneum* and lowest in *Cylindrospermum muscicola*. Algae with brighter thalli were reported to be rich in vitamin C (44). In the present study, *Oscillatoria subbrevis* and *Cylindrospermum muscicola* were found to colonize with brighter blue-green and olive green colour thalli on polythene bags. *Oscillatoria subbrevis* formed bright blue-green loop like thallus floating over the liquid medium and *Cylindrospermum muscicola* formed olive green finger like projection on the petri plate surface in laboratory culture.

Conclusion

Five species of cyanobacteria isolated from submerged polythene surface in domestic sewage water are demonstrated to be a rich source of carbohydrate, proteins, lipids, vitamin C and phycobiliproteins.The results are anticipated to be of relevance to biodegradation of polythenes, aquaculture, pharmaceutical

Table 3: Biochemical composition of five cyanobacterial isolates from submerged polythene bags in domestic sewage water

Biochemical parameters	Cyanobacterial isolates					
	Phormidium lucidum	*Oscillatoria subbrevis*	*Lyngbya diguetii*	*Nostoc carneum*	*Cylindros permum muscicola*	CD (0.05%)
Chl*a* (µgml^{-1}	8.47±0.12	7.32±0.23	6.92±0.34	3.02±0.21	4.23±0.42	0.02
Carotenoid (µgml^{-1})	2.10±0.02	2.89±0.01	1.89±0.03	0.89±0.01	1.02±0.02	0.01
Protein(µgml^{-1})	210±o.54	230±0.51	203±0.32	195±0.43	145±0.34	0.02
Carbohydrate (µgml^{-1})	240±0.73	370±1.02	230±0.45	113±0.56	109±0.67	0.04
Vitamin C (µgml^{-1})	0.5±0.01	0.9±0.03	0.8±0.01	0.9±0.01	0.3±0.01	0.02
Lipid (µgml^{-1})	8.7±0.02	11.2±0.21	7.3±0.03	5.1±0.12	4.2±0.12	0.04

Table 4: Phycobiliproteins of five cyanobacterial isolates from submerged polythene bags in domestic sewage water

Phycobiliproteins	Cyanobacterial isolates					
	Phormidium lucidum	*Oscillatoria subbrevis*	*Lyngbya diguetii*	*Nostoc carneum*	*Cylindros permum muscicola*	CD (0.05%)
PE(µgml^{-1})	14.2±0.12	13.1±0.21	17.5±0.12	12.1±0.21	15.3±0.34	0.02
PC(µgml^{-1})	42.4±0.02	48.7±0.23	36±0.23	45±0.04	15.34±0.15	0.01
APC(µgml^{-1})	18±0.01	19.6±0.12	25±0.05	21±0.24	15.34±0.02	0.04
Total Phycobiliproteins (µgml^{-1})	74.6±0.15	81.4±0.56	78.5±0.4	78.1±0.49	45.98±0.51	0.05

applications and biofuel.Due to rich biochemical contents these cyanobacteria may have the potential for use in the food industry as high value nutritional products.

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Identification of Nitrogen Efficient Indica Rice (*Oryza sativa* L.) Genotypes: A physiological and Multivariate statistical approach

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Abstract

Nitrogen efficiency (NE) is an important approach to develop nitrogen use efficient (NUE) genotypes in rice growing regions globally. Identification of nitrogen (N) tolerant genotype is the foremost step to develop Nitrogen Use Efficient (NUE) genotypes. In order to identify the N tolerant genotypes in popular indica rice a hydroponic experiment was conducted in glass house with various concentrations of N and 26 genotypes were screened by measuring growth dynamics, biomass accumulation and NE traits. The data revealed that growth dynamics *viz.,* morphological (TNL, RL and SL), biomass (RFW, SFW, RDW, SDW and TDM) and NE traits showed high genotypic variation for N treatments. Of which root and shoot traits were significantly influenced the nitrogen efficiency trait of genotypes. Based on NE; genotypes were categorized as High Efficient; Medium efficient and Low efficient genotypes. Growth dynamics and NE traits were higher in DRRH3 at low N condition; while BPT5204 responds very poor under low N which confirmed that DRRH3 as most N-efficient and BPT5204 as most N-inefficient genotypes. Further experimental data was subjected to multivariate statistical analysis by means of Pearson correlation, PCA and hierarchical clustering of heatmap analysis. The statistical data is in tune with the experimental data and strongly supported that root and shoot traits are most contributing traits for NE of genotypes. The data generated in the present study is very useful for the selection of genotypes as parents for the development of NUE genotypes of rice.

Key words: Rice genotypes, N treatments, growth dynamics, Nitrogen efficiency, Multivariate statistical analysis, N-efficient and N-inefficient genotypes.

Introduction

Rice is a staple food crop of the world; sixty percent of the global population depends on it for more than 22% of their daily calories (20). Ninety percent of the global rice is produced especially from Asian countries *i.e.,* China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Pakistan, Philippines, Korea and Japan. Among the rice growing countries India occupies largest place with 44.8 million hectares of cultivation followed by China and Indonesia. Over the past half-century a marked increase in rice production attributed due to high yielding genotypes, usage of more folds of N-chemical fertilizers and modern agricultural techniques (25).

To steady the food demands; increased use of N- fertilizer content in the world from 3.5 million metric tons in 1960 to projected 187.7 million metric tons in 2015 and 223.1 million metric tons by 2030 (37). A major portion of global N-fertilizer would be used for the production of the rice (42). Rice based cropping system is one the most

incompetent N-user; only 30% - 50% of applied N-fertilizer utilized and in some cases less than it (27)(31); unutilized N-fertilizer dissipates by leaching, de nitrification, immobilization and volatilization (40) resulted drastic effect on environment and all living life forms (6) (11) (28) (33) (39). The efficient absorption and utilization of nitrogen is the key factor for the development high yielding varieties in rice. As the nutrient uptake and utilization are co-dependent careful integration of both physiological and agronomic traits are very important to develop nitrogen efficient genotypes.

Genetic variability for efficiency in N-absorption and utilization have been documented in several crops (18). Development of a reliable selection criterion is very important for identification of genetic variability Nitrogen Use Efficient (NUE) genotypes. This will confirmly help us to enhance the nitrogen uptake and utilization efficiency which eventually leads to the Nitrogen Use Efficiency in crop plants (17).

Hydroponics is a foremost controlled and elementary modeling tool for plant research, it is also denoted as 'water culture of plants' and has been widely used in research as well as in commercial farming since from 18th century onwards. Hydroponics is very important for plant research as it helps to identify essential elements, their optimum levels and plant uptake form of element under well controlled nutrient solutions. It also helpful for the identification of intra and inter specific genetic variations with respect to different treatment levels and to identify the elements deficiency, toxicity symptoms under more controlled conditions (34).

To determine the limits of concentration of nitrogen in rice genotypes is an important aspect as the rate of absorption and assimilation of applied nitrogen varies in the genotypes. Thus the present study aimed to screen the rice genotypes for their nitrogen efficiency with different nitrogen concentrations as KNO_3 as source of nitrogen in a hydroponic experiment. This preliminary screening experiment would identify the Nitrogen Use Efficient genotypes which would be further helpful to the breeders for development of high nitrogen efficient genotypes.

Materials and methods

Genotypes for study : Twenty six rice (***Oryza sativa* L.**) genotypes *viz.,* KMR3R, RPHR-1096, DRRH3, B-95-1, RPHR-111-3, AJAYA-R, , EPLT-104, DR714-1-2R, SC5-2-2-1, KRH2, BCW-56, RPHR-1005 and EPLT-109 were supplied by the Directorate of Rice Research (DRR), Rajendranagar, Hyderabad, RAMAPPA, JAYA, JGL-MASURI, BADRAKALI, NLR-3042, WGL-347, JGL-1798, SWARNA, MTU1010, BPT5204, ERRAMALLELU and IMPROVED SAMBA MASURI were supplied by the Agricultural research station, Utukuru, Kadapa and BI 33 was supplied by the GKVK, Bangalore.

Experimental design : The experiment was conducted in a completely randomized block design (CRBD) with two replicates. Four levels of nitrogen *viz.,* nitrogen deficiency/T1 treatment (0 mM L^{-1}), low nitrogen/T2 treatment (1 mM L^{-1}), medium nitrogen/T3 treatment (4 mM L^{-1}) and high nitrogen/T4 treatment (10 mM L^{-1}) were supplied in the form of potassium nitrate (KNO_3). Surface sterilized seeds (0.05% $HgCl_2$) were germinated in germination boxes on filter paper for one week. After, similar sizes of seedlings were placed in nutrient solution (17). containing macronutrients of 5.6 mM L^{-1} K_2SO_4, 3.4 mM L^{-1} $CaCl_2.2H_2O$, 0.9 mM L^{-1} $MgSO_4.7H_2O$, 0.9 mM L^{-1} NaH_2Po_4 and micro nutrients of 21.5 mM L^{-1} $FeCl_3.6H_2O$, 23.0 mM L^{-1} H_3BO_3, 9 mM L^{-1} $MnCl_2.4H_2O$, 0.3 mM L^{-1} $(NH_4)6Mo_7O_{24}.4H_2O$, 0.9 mM L^{-1} $CuSO_4.4H_2O$, 3.5 mM L^{-1} $ZnSO_4.7H_2O$ in the culture room maintained at 26/22^{oC} day/night temperature, 60% relative humidity and 16/8 hrs light/dark photoperiod with 2650 lumens of output light intensity. The nutrient solution was aerated with sterile air to provide sufficient O_2 and changed every week up to 30 days.

Experimental observations

Growth dynamics (GD) : Growth dynamics were separated into morphological and biomass traits.

Morphological traits : Plant samples were taken out from nutrient solution and separated in to root

and shoot. The morphological traits were partitioned into total number of leaves (TNL), root length (RL) and shoot length (SL). The root length was measured from the root-shoot junction to tip of the longest root and shoot length was measured from the soil above ground level up to uppermost longest fully expanded leaf (1). The root and shoot lengths were measured by using metric scale and expressed in terms of centimeters (cm).

Biomass traits *:* Biomass was partitioned into root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW), shoot dry weight (SDW) and total dry matter (TDM). For fresh weights, root and shoot samples were dried with blotting sheets and recorded by using electronic weighing balance (Model AX200, SHIMADZU, JAPAN) and for dry weights plant samples were dried at 65^{oC} for 72 hrs in hot air oven (proper care taken to avoid burning of the samples) and recorded by using electronic weighing balance (Model AX200, SHIMADZU, JAPAN). The fresh and dry weights were expressed in terms of grams (g) per plant.

Nitrogen efficiency (NE) : Nitrogen efficiencies of 26 rice genotypes were calculated based on their mean of shoot dry weight values, which were applied into following formula according to Chandna et al. (2010) (8) and expressed in terms of percentage (%).

Nitrogen efficiency (%) = (Biomass accumulation at N-insufficient condition / Biomass accumulation at N-sufficient condition) x 100

Multivariate statistical analysis : All data were subjected to statistically analyzed by means of experimental treatments average mean to growth dynamics and nitrogen efficiency traits for observing response with different nitrogen levels and multivariate statistical analysis by means of Pearson correlation coefficient (19) for relation of traits with respect to different nitrogen levels among genotypes, principal component analysis (PCA) (32) for identification of most contributing of traits for higher nitrogen efficient genotypes under low nitrogen levels and finally done the hierarchical heatmap and cluster analysis (32) for better screening of nitrogen efficient genotypes with graphical representation and genotypes grouping with respect to different nitrogen treatment levels.

Results

N-treatment effect on growth dynamics (GD) : Effect of various concentrations of nitrogen on growth dynamics (GD) and nitrogen efficiency (NE) of 26 rice genotypes were assessed and genotypes were categorized using multivariate statistical analysis by means of Pearson correlation coefficient, principal component analysis (PCA) and hierarchical heatmap clustering. The growth dynamics *i.e.,* total number of leaves (TNL), root length (RL), shoot length (SL), root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW), shoot dry weight (SDW) and total dry matter (TDM) displayed high genotypic variations across nitrogen treatments. In the present study, the concentration of nitrogen frailly affects the growth and development of leaves. However, a significant genotypic variation observed for total number of leaves (TNL/P) with a mean data ranged from 1.95 (BPT5204) to 3.58 (DRRH3) under various nitrogen treatments and an average of greater number of leaves 4.0 (TNL/P) were observed in the genotype DRRH3 at medium N rate (4 mM L^{-1}) and fewer number leaves in genotype BPT5204 at N deficient (0 mM L^{-1}) condition. Root length sturdily affected by the N treatment. Mean root lengths of genotypes varied from 2.24 cm (BPT5204) to 9.16 cm (DRRH3), with an average value of 9.58 cm in genotype DRRH3 at medium N rate (4 mM L^{-1}) and 1.50 cm by the RPHR-111-3 at high N rate (10 mM L^{-1}). In contrast high root fresh weight (RFW) was recorded in DRRH3 (0.025 g) and lowest in B-95-1 (0.007 g). The root dry weight (RDW) was ranged from 0.002 g (B-95-1, AJAYA-R, EPLT-104 and BPT5204) to 0.025 g (DRRH3) observed at T2 and T3 levels and with a mean data the highest RDW was recorded in DRRH3 (0.019 g) and lowest in BPT5204 (0.003 g).

The shoot lengths were significantly affected by the N treatments and mean shoot lengths varied from 3.68 cm (BPT5204) to 10.05 cm (DRRH3).

The genotype JGL-MASURI recorded an average of 13.00 cm at high N level (10 mM $L^{-1)}$ and 2.90cm by BPT5204 at low N levels (0 mM L^{-1}). Shoot fresh weights (SFW) were ranged from 0.004 g (B-95-1) to 0.052 g (EPLT-104) observed at T1 levels and with a mean data the highest SFW was recorded in EPLT-104 (0.025 g) and lowest in B-95-1 (0.006 g). The shoot dry weight (SDW) were ranged from 0.002 g (B-95-1, EPLT-104 and BPT 5204) to 0.015 g (JAYA) observed at T1, T2 and T3 levels and with a mean data the highest SDW was recorded in JAYA (0.012 g) and lowest in B-95-1 (0.003 g). The total dry mater (TDM) was ranged from 0.004 g (B-95-1 and BPT5204) to 0.032 g (DRRH3) observed at T2 level and with a mean data the highest TDW was recorded in DRRH3 (0.028 g) and lowest in B-95-1 (0.007 g) (Table 1).

Simple correlation data analysis revealed the relationship between traits among genotypes with respect to nitrogen treatments. RL significantly correlated with TNL/P ($p < 0.01$) and SL ($p < 0.05$) with a correlation coefficient (r) of 0.610, 0.424 respectively. RFW was highly significant ($p < 0.01$) with RL and SL with a correlation coefficient (r) of 0.564 and 0.422 respectively, SFW was highly significant ($p < 0.01$) with RFW with a correlation coefficient (r) of 0.576, RDW was highly significant ($p < 0.01$) with RL and RFW with correlation coefficients (r) of 0.553 and 0.698 respectively, SDW was positive significant with all measured traits, TDM was highly significant ($p < 0.01$) with RL, SL, RFW, RDW and SDW significantly ($p < 0.05$) correlated with TNL with a correlation coefficients (r) of 0.624, 0.580, 0.676, 0.933, 0.914 and 0.415 respectively (Table 2 a-d).

Treatments effect on nitrogen efficiency (NE): Screening of genotypes to nitrogen tolerance was determined based on the nitrogen efficiency (NE), which is derived from the shoot dry weight NE is a widely considered parameter for identification of genotypic variation in tolerance to nutrient deficiency (2). In the present study, NE data displayed high genotypic variation under different nitrogen regimes. NE values were ranged from 22.22% to 200.00% at T3 treatment (4 mM L^{-1}) and 20.00% to 166.67% T4 treatment (10 mM L^{-1}). This data clearly indicates that plants were unable to up take the excess N source. At medium nitrogen conditions NE significantly positively correlated with root dry weight (RD) ($p < 0.01$) and root length (RL) ($p < 0.05$) and negatively correlated with shoot dry weight (SDW) ($p < 0.01$) with correlation coefficients (r) of 0.499; 0.457 and -0.545 respectively. Similarly, NE at high nitrogen (10 mM L^{-1}) conditions had significant positive correlation with RDW ($p < 0.05$), RL ($p < 0.01$) and negative correlation with SDW ($p < 0.05$) with correlation coefficients (r) of 0.491; 0.526, and -0.486 respectively. It indicates both at medium and high nitrogen conditions root length and root dry weight are key contributing traits for genotypes NE. Further, as per Hakeem et al. (2012) (17), based on the NE data at both N treatments (4 mM L^{-1} and 10 mM L^{-1}) in the present study genotypes were grouped into three categories viz., high nitrogen efficient (HNE) genotypes (the genotypes contained more than 90% of NE values), moderate nitrogen efficient (MNE) genotypes (the genotypes contained between 65.00-89.99% of NE values) and low nitrogen efficient (LNE) genotypes (the genotypes contained below 65.00% of NE values). Accordingly, KMR3R, RPHR-196, DRRH3, NLR-3042, SC5-2-2-1, KRH2 and SWARNA were grouped as high nitrogen efficient (HNE) genotypes, the RPHR-111-3, BI33, RAMAPPA, BCW-56, RPHR-1005, JAYA, JGL-MAHSURI, MTU-1010, BADRAKALI, ERRAMALLELU and IMPROVED SAMBA MAHSURI were grouped as moderate nitrogen efficient (MNE) genotypes and the B-95-1, AJAYA-R, EPLT-104, DR714-1-2R, WGL-347, EPLT-109, JGL-1798 and BPT5204 were grouped as low nitrogen efficient (LNE) genotypes. Overall at T3 and T4 treatments DRRH3 considered as high nitrogen efficient genotype and the BPT 5204 as low nitrogen efficient genotype (Table 1).

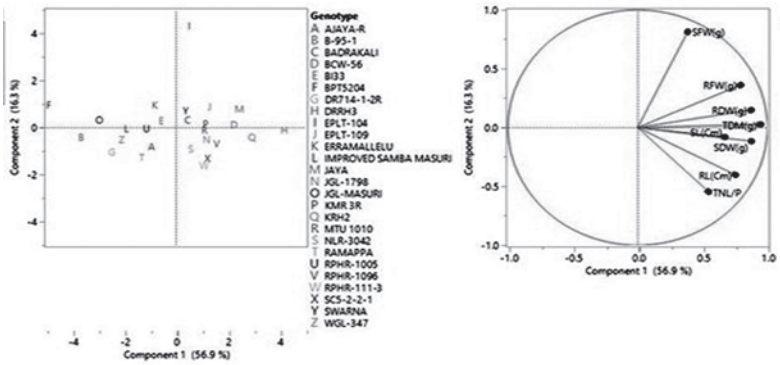
Principal Component Analysis (PCA) : Principal Component Analysis (PCA) was performed to accurately identify the most contributing traits in categorizing the genotypes in response different nitrogen treatments. At deficient nitrogen (0 mM L^{-1}) condition the PCA of first four components contributed 90.325% of

variability and rest of the components contributed 9.676% of variability among genotypes for growth dynamic traits. Principal component PC 1 had the Eigen value 4.550 and contributed 56.880% of total variability which is obtained from the traits such as TDM (0.445), SDW (0.412), RDW (0.411), RFW (0.373), RL (0.354), SL (0.316) and TNL (0.257), the PC 2 had Eigen value 1.301 and contributed 16.261% of total variability obtained SFW (0.716) and RFW (0.317), the PC 3 had Eigen value 0.792 and contributed 9.897% of total variability obtained from TNL (0.509), RL (0.286), RFW (0.280) and SFW (0.237) and the PC 4 had Eigen value 0.583 and contributed 7.287% of total variability obtained from SL (0.622), TNL (0.355), SFW (0.354) and RL (0.286). TDM, SDW and RDW of PC 1, SFW of PC 2, TNL of PC 3 and SL of PC 4 were had the high positive values indicated more contribution towards total variability. At deficient nitrogen (0 mM L^{-1}) condition based on PCA analysis high ranking genotypes DRRH3, DR714-1-2R, JAYA, BCW-56 and SWARNA considered as high tolerant genotypes and low ranking genotypes BPT 5204, B-95-1, JGL-MAHSURI, DR714-1-28, WGL-347 and EPLT-104 considered as more sensitive genotypes to nitrogen deficiency conditions (Figure 1a).

At low nitrogen (1 mM L^{-1}) condition the PCA of first four components contributed for 90.457% of genotypic variability and rest of the components were for 9.543% of variability. Principal component PC 1 had the Eigen value 5.217 and contributed 65.210% of total variability obtained from TDM (0.408), SFW (0.383), RDW (0.378), RFW (0.366), SDW (0.345), RL (0.324), TNL (0.315) and SL (0.295), the PC 2 had Eigen value 0.804 and contributed 10.047% of total variability obtained from major contribution of SL (0.555), TNL (0.460), SFW (0.224) and RL (0.207), the PC 3 had Eigen value 0.642 and contributed 8.021% of total variability obtained from RL (0.614), RFW (0.335) and RDW (0.249) and the PC 4 had Eigen value 0.574 and contributed 7.179% of total variability obtained from major contribution of SL (0.638) and RFW (0.349). TDM of PC 1, SL and TNL of PC 2, RL of PC 3 and SL of PC 4 were had the high positive values indicated more contribution towards total variability. At deficient nitrogen (1 mM L^{-1}) condition based on PCA analysis genotype were grouped into two categories, high tolerant genotypes DRRH3, KRH2, DR714-1-28, BI 33 and JAYA with high higher ranking values, and sensitive genotypes BPT5204, B-95-1, AJAYA-R, EPLT-104, WGL-347, JGL-1798 and RAMAPPA with low PC values (Figure 1b).

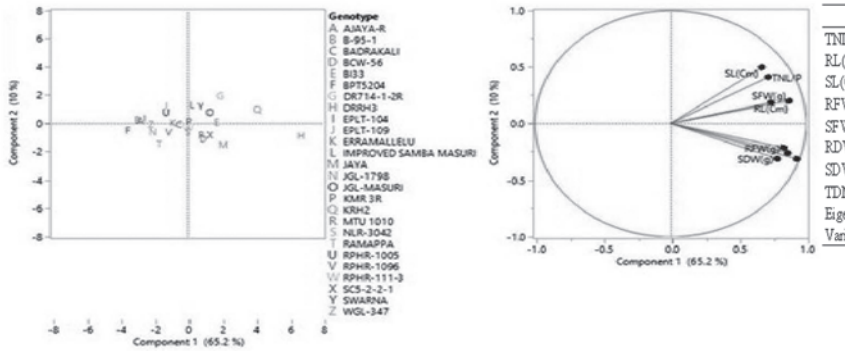
At medium nitrogen (4 mM L^{-1}) condition 88.593% of genotypic variability contributed by the first four components of PCA and 11.407% of variability by the rest of the components. PC 1 had the Eigen value 4.438 and contributed 49.307% of total variability obtained from the traits RDW (0.422), TDM (0.418), TNL (0.414), RL (0.375), RFW (0.351), SL (0.338), NE (0.238) and SFW (0.204), the PC 2 had Eigen value 2.064 and contributed 22.937% of total variability obtained from SDW (0.638) and SFW (0.538), the PC 3 had Eigen value 0.816 and contributed 9.065% of total variability obtained from SL (0.655), RL (0.255) and TNL (0.201) and the PC 4 had Eigen value 0.656 and contributed 7.284% of total variability obtained from RFW (0.640), SL (0.385) and NE (0.332). RDW, TDM and TNL of PC 1, SDW and SFW of PC 2, SL of PC 3 and RFW of PC 4 were had the high positive values indicated more contribution towards total variability. Genotypes based the PCA values high ranking value genotypes such as DRRH3, KRH2 and BI33 considered as high tolerant genotypes and low PCA value genotypes BPT 5204, B-95-1, WGL-347, RPHR-1005 and JGL-1798 considered as more sensitive genotypes to moderate nitrogen levels (Figure 1c).

At high nitrogen (10 mM L^{-1}) condition the PCA of first four components contributed 88.947% of genotypic variability and rest of the components 11.053% of variability. PC 1 had the Eigen value 4.747 and contributed 52.746% of total variability obtained from TDM (0.424), TNL (0.378), RDW (0.375), RFW (0.361), SFW (0.341), SL (0.325) and SDW (0.207), the PC 2 had Eigen value 2.004 and contributed 22.269% of total variability obtained from NE (0.589), RL (0.312) and RDW (0.292),



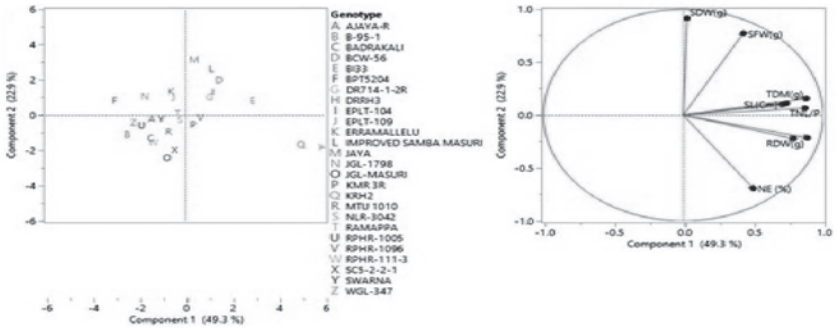
	PC1	PC2	PC3	PC4
TNL/P	0.257	-0.479	0.509	0.355
RL(Cm)	0.354	-0.353	0.286	0.041
SL(Cm)	0.316	-0.071	-0.599	0.622
RFW(g)	0.373	0.317	0.280	0.092
SFW(g)	0.182	0.716	0.237	0.354
RDW(g)	0.411	0.131	0.072	-0.464
SDW(g)	0.412	-0.102	-0.373	-0.144
TDM(g)	0.445	0.024	-0.148	-0.340
Eigenvalue	4.550	1.301	0.792	0.583
variability	56.880	16.261	9.897	7.287

Fig. 1(a). Principal component analysis for various growth dynamic traits in 26 rice genotypes under nitrogen deficient condition.



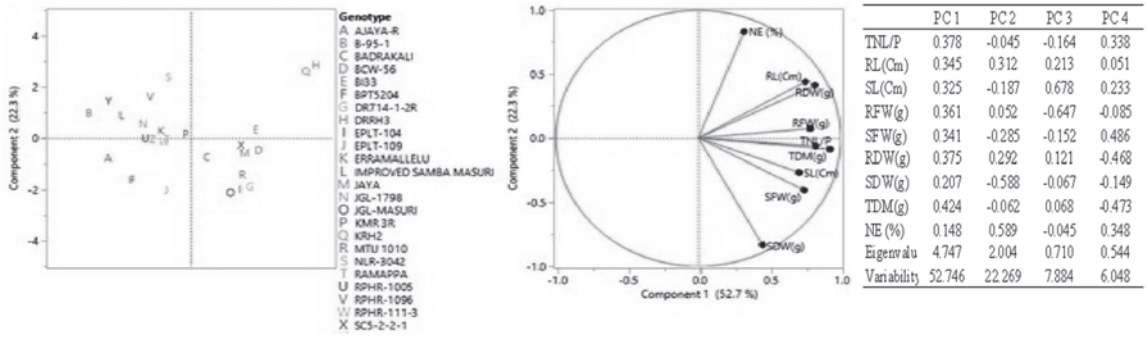
	PC 1	PC 2	PC 3	PC 4
TNL/P	0.315	0.460	-0.085	-0.665
RL(Cm)	0.324	0.207	0.614	-0.096
SL(Cm)	0.295	0.555	-0.137	0.638
RFW(g)	0.366	-0.241	0.335	0.349
SFW(g)	0.383	0.224	-0.337	-0.008
RDW(g)	0.378	-0.292	0.249	-0.118
SDW(g)	0.345	-0.348	-0.552	0.011
TDM(g)	0.408	-0.351	-0.071	-0.076
Eigenvalu	5.217	0.804	0.642	0.574
Variability	65.210	10.047	8.021	7.179

Fig. 1(b). Principal component analysis for various growth dynamic traits in 26 rice genotypes under low nitrogen condition.



	PC 1	PC 2	PC 3	PC 4
TNL/P	0.414	0.047	0.201	-0.258
RL(Cm)	0.375	-0.154	0.255	-0.294
SL(Cm)	0.338	0.072	0.655	0.117
RFW(g)	0.351	0.079	-0.371	0.640
SFW(g)	0.204	0.538	0.183	0.385
RDW(g)	0.422	-0.150	-0.349	-0.199
SDW(g)	0.013	0.638	-0.130	-0.217
TDM(g)	0.418	0.110	-0.394	-0.282
NE (%)	0.238	-0.481	0.026	0.332
Eigenvalue	4.438	2.064	0.816	0.656
ariability (%	49.307	22.937	9.065	7.284

Fig.1(c). Principal component analysis for various growth dynamic and nitrogen efficiency traits in 26 rice genotypes under moderate nitrogen condition.



	PC 1	PC 2	PC 3	PC 4
TNL/P	0.378	-0.045	-0.164	0.338
RL(Cm)	0.345	0.312	0.213	0.051
SL(Cm)	0.325	-0.187	0.678	0.233
RFW(g)	0.361	0.052	-0.647	-0.085
SFW(g)	0.341	-0.285	-0.152	0.486
RDW(g)	0.375	0.292	0.121	-0.468
SDW(g)	0.207	-0.588	-0.067	-0.149
TDM(g)	0.424	-0.062	0.068	-0.473
NE (%)	0.148	0.589	-0.045	0.348
Eigenvalu	4.747	2.004	0.710	0.544
Variability	52.746	22.269	7.884	6.048

Fig. 1(d). Principal component analysis for various growth dynamic and nitrogen efficiency traits in 26 rice genotypes under high nitrogen condition.

the PC 3 had Eigen value 0.710 and contributed 7.884% of total variability obtained from SL (0.678) and RL (0.213), and the PC 4 had Eigen value 0.544 and contributed 6.048% of total variability obtained from SFW (0.486), NE (0.348), TNL (0.338) and SL (0.233). At higher nitrogen levels TDM, NE, SL and SFW contributed more for the genetic variability. High PCA value genotypes such as DRRH3, KRH2, BI33, BCW-56, DR714-1-2R, SC5-2-2-1, JAYA, MTU1010, EPLT-104 and JGL-MAHSURI were considered as high tolerant genotypes and low PCA value genotypes B-95-1, SWARNA, AJAYA-R, IMPROVED SAMBA MAHSURI, BPT 5204, JGL-1798, RPHR-1005 and WGL-347 were considered as sensitive to high nitrogen levels (Figure 1d).

Hierarchical heatmap and cluster analysis

Hierarchical heatmap and cluster analysis was carried out in twenty six genotypes to get an accurate confirmation on categorization of genotypes based on growth dynamics and NE traits. Fine graphical representations are very useful to examine the genotypes and their contributed traits for judgment of the experimental results.

Under deficient nitrogen source (0 mM L^{-1}) the twenty six rice genotypes mainly divided in to two clusters viz., cluster I and cluster II, they again sub divided into two sub-clusters individually *viz.,* cluster 1A, cluster 1B and cluster II A, cluster II B. Fourteen genotypes grouped in cluster I A *viz.,* KMR3R, EPLT-109, SWARNA, BADRAKALI, RPHR-1096, BCW-56, DRRH3, KRH2, JAYA, RPHR-111-3, SC5-2-2-1, NLR-3042, JGL-1798 and MTU1010, which showed maximum response to low nitrogen conditions, only one genotype was there in cluster I B *viz.,* EPLT-104 performed well but comparatively low to class IA under low nitrogen conditions, genotypes under cluster II A *viz.,* B-95-1, JGL MAHSURI and BPT5204 responded poorly and cluster II B genotypes *viz.,* AJAYA-R, RAMAPPA, DR714-1-2R, WGL-347, IMPROVED SAMBA MAHSURI, BI33, RPHR-1005 and ERRAMALLELU performed moderately under nitrogen deficit conditions. The cluster analysis data confirmed that cluster I A and I B genotypes were considered as high tolerant genotypes, cluster II A genotypes considered as high sensitive genotypes and cluster II B genotypes considered as moderate genotypes at nitrogen deficient conditions. In this treatment, the contributed traits were distinguished into three groups *viz.,* group I, group II and group III. The group I have morphological traits of SL, RL and TNL, group II have biomass traits of SDW, TDM and RDW and group III have biomass traits of RFW and SFW. NE trait wasn't considered in this grouping for this treatment because there is no statistical value (Figure 2a).

Under low nitrogen source (1 mM L^{-1}) the twenty six rice genotypes were mainly divided in to two clusters *viz.,* cluster I and cluster II, cluster I again sub divided into two sub-clusters *viz.,* cluster 1A, cluster 1B. There were 18 genotypes

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grouped into cluster I A *viz.,* KMR3R, SC5-2-2-1, RPHR-1096, RAMAPPA, DR714-1-2R, SWARNA, IMPROVED SAMBA MAHSURI, RPHR-1005, ERRAMALLELU, BADRAKALI, EPLT-109, RPHR-111-3, NLR-3042, JGL MAHSURI, MTU1010, BI33, BCW-56 and JAYA, DRRH3, KRH2 which exhibited moderate response to nitrogen, genotypes in the cluster I B *viz.,* B-95-1, AJAYA-R, EPLT-104, WGL-347, BPT5204 and JGL-1798 displayed poor response and cluster II remains in single group contained DRRH3 and KRH2 genotypes which performed very well and displayed maximum response under low nitrogen level. This data clearly established that cluster I A genotypes as moderate genotypes, cluster I B genotypes as sensitive genotypes and cluster II genotypes as tolerant genotypes at low nitrogen level. Under low nitrogen condition the contributed traits were distinguished into two group's *viz.,* group I and group II. The group I have morphological traits of SL, RL and TNL. NE wasn't considered in this group because as there is no statistical value. Group II have biomass traits of RFW, SDW, SFW, TDM and RDW (Figure 2b).

At moderate nitrogen source (4 mM L^{-1}) the twenty six rice genotypes were mainly divided in to two clusters *viz.,* cluster I and cluster II, they again sub divided into two sub-clusters individually *viz.,* cluster 1A, cluster 1B and cluster II A, cluster II B. The cluster I A contains genotypes *viz.,* KMR3R, RPHR-1096, RAMAPPA, JGL MAHSURI, RPHR-111-3, SC5-2-2-1, NLR-3042, MTU1010 and SWARNA which showed moderate response to nitrogen and cluster I B contains genotypes *viz.,* B-95-1, AJAYA-R, RPHR-1005, BADRAKALI, WGL-347, BPT5204, EPLT-109, ERRAMALLELU and JGL-1798 which are very poor responders to nitrogen at this concentration. The genotypes in the cluster II A *viz.,* DRRH3 and KRH2 II B are high responsive genotypes to medium nitrogen concentration, genotypes of cluster II B such as BI33, EPLT-104, IMPROVED SAMBA MAHSURI, DR714-1-2R, BCW-56 and JAYA performed well at this concentration but comparatively lesser than group II A. This data clearly confirmed that cluster I A genotypes considered as moderately tolerant

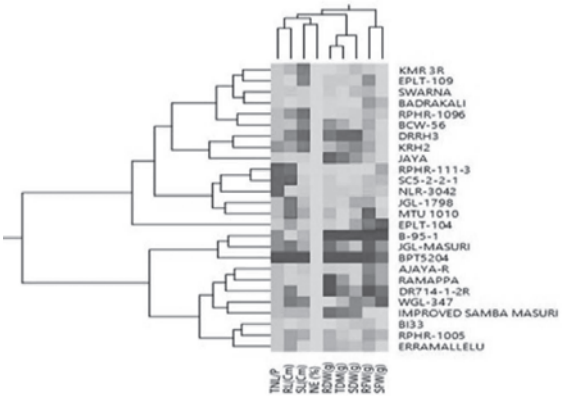

Fig. 2(a). Hierarchical heatmap clustering for various growth dynamic traits in 26 rice genotypes under nitrogen deficient condition.

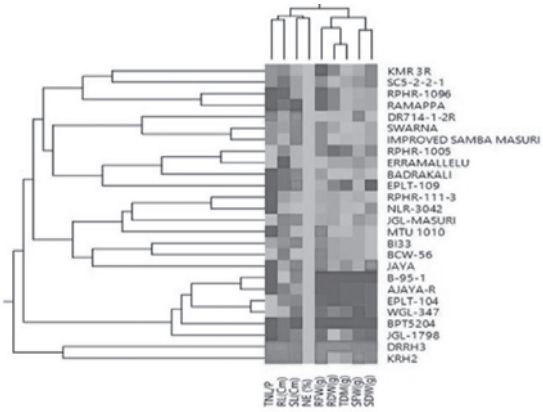

Figure 2(b). Hierarchical heatmap clustering for various growth dynamic traits in 26 rice genotypes under low nitrogen condition.

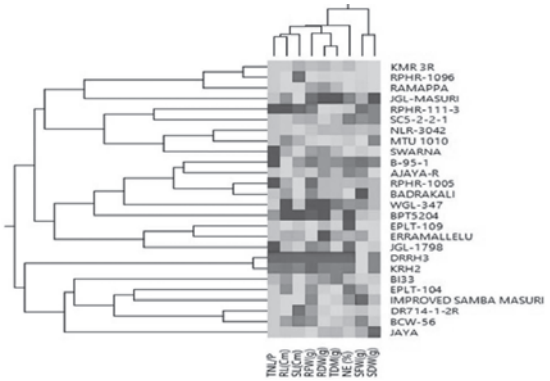

Fig. 2(c). Hierarchical heatmap clustering for various growth dynamic and nitrogen efficiency traits in 26 rice genotypes under moderate nitrogen condition.

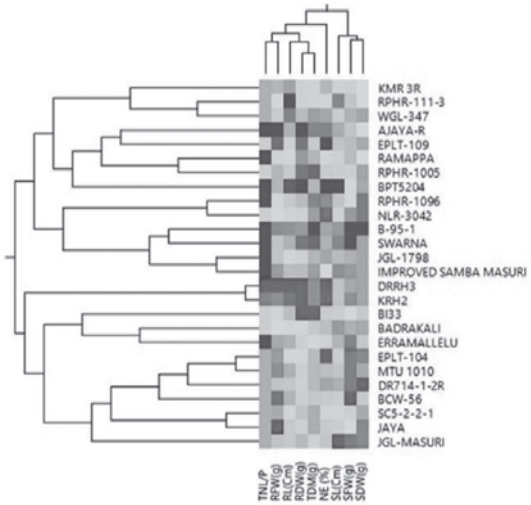

Fig. 2(d). Hierarchical heatmap clustering for various growth dynamic and nitrogen efficiency traits in 26 rice genotypes under high nitrogen condition.

and cluster I B genotypes as sensitive, cluster II A genotypes as highly tolerant and cluster II B genotypes considered as tolerant at moderate nitrogen level. In this treatment, the contributed traits were distinguished into four group's *viz.,* group I, group II, group III and group IV. The group I have morphological traits of SL, RL and TNL, group II have biomass traits of RFW, SDW, RDW and TDM, group III has NE and group IV have SFW and SDW (Figure 2c).

At high nitrogen source (10 mM L^{-1}) twenty six rice genotypes were mainly grouped in to two clusters *viz.,* cluster I and cluster II, they again sub divided into two sub-clusters individually *viz.,* cluster 1A, cluster 1B and cluster II A, cluster II B. Cluster I A consisting of KMR3R, RPHR-111-3, WGL-347, AJAYA-R, EPLT-109, RAMAPPA, RPHR-1005 and BPT5204 which displayed moderate response to high nitrogen levels, cluster I B genotypes *viz.,* RPHR-1096, NLR-3042, B-95-1, SWARNA, JGL-1798 and IMPROVED SAMBA MAHSURI., exhibited less performance, cluster II A genotypes *viz.,* DRRH3 and KRH2 performed very well and high response to high nitrogen levels and cluster II B *viz.,* BI33, BADRAKALI, ERRAMALLELU, EPLT-104, MTU 1010, DR714-1-2R, BCW-56, SC5-2-2-1, JAYA and JGL MAHSURI performed well but comparatively lesser than group II A. This data clearly confirmed that cluster I A genotypes considered moderate and cluster I B genotypes were considered as sensitive, cluster II A genotypes were considered as high tolerant and cluster II B genotypes were considered as tolerant at moderate nitrogen level. In this treatment, the contributed traits were distinguished into three groups *viz.,* group I, group II and group III. The group I have RFW, TDM, RDW, RL and TNL, group II have only NE and group III have SFW, SDW and SL (Figure 2d).

Discussion

Nitrogen is an essential element for growth and other physiological functions of the plant body. The amount of nitrogen can influence the absorption of light, light use efficiency and accumulation of dry matter in various parts of the plants (5). In general, plants absorb and utilize nutrients very rapidly at their early stages of growth as the availability of nutrients are very high in the soil. Genotypes differently absorb and utilize the nutrients. Differential absorption of nutrients by the genotypes depends on several factors such as genotype nature, size and morphology of the root, nutrients requirement, availability of nutrients, growth stage of the plant, uptake and allocation efficiency of the genotype and nutrient use efficiency (26). Though it is unwarranted, several crop plants unavoidably uptake excess nitrogen fertilizer from the soil for their growth and development. However, only 50% or less of the absorbed nitrogen is used and rest of the nitrogen vanished into the environment and cause severe environmental pollution. To overcome this, identification of N-efficient genotypes which can absorb and efficiently utilize the accumulated high nitrogen or genotypes which can grow and yield under low N conditions are very important in the agronomical context. In a previous study based the nitrogen uptake kinetics and biochemical analysis several rice genotypes were screened and identified high NE genotypes. The high NE genotypes had greater amount of nitrogen contents than low NE genotypes (8) (16).

"Table 1. Effects of nitrogen treatments on growth dynamics and nitrogen efficiency of the tested rice genotypes".

N treatment	KMR 3R	RPHR-1096	DRRH3	B-95-1	RPHR-111-3	NLR-3042	AJAYA-R	BB3	EPLT-104	DRR14-1-2R	SC5-2-2-1	WGL-347	RAMAPPA	KRH2
Total number of leaves														
0 mM L^{-1}	2.5 ± 0.50	3.0 ± 0.40	3.0 ± 0.20	2.0 ± 0.10	3.5 ± 0.50	3.5 ± 0.50	3.0 ± 0.00	2.5 ± 0.40	2.5 ± 0.50	3.0 ± 1.0	3.5 ± 0.50	3.0 ± 1.0	3.0 ± 1.0	3.2 ± 0.50
1 mM L^{-1}	3.0 ± 0.50	2.0 ± 0.50	3.8 ± 0.50	2.0 ± 0.30	2.0 ± 0.50	2.0 ± 0.00	2.0 ± 0.00	2.8 ± 0.50	2.5 ± 0.50	3.5 ± 0.50	3.0 ± 1.0	2.5 ± 0.50	2.0 ± 0.00	3.5 ± 0.50
4 mM L^{-1}	3.0 ± 0.80	3.0 ± 0.30	4.0 ± 0.30	2.0 ± 0.20	2.0 ± 0.40	2.5 ± 0.50	2.5 ± 0.50	3.2 ± 0.80	3.0 ± 0.00	3.0 ± 0.00	2.5 ± 0.50	3.0 ± 1.0	3.0 ± 1.0	3.8 ± 0.40
10 mM L^{-1}	3.0 ± 0.40	2.0 ± 0.20	3.5 ± 0.40	2.0 ± 0.50	3.0 ± 1.0	2.5 ± 0.50	2.0 ± 0.00	2.5 ± 0.40	3.0 ± 0.00	3.0 ± 0.00	3.0 ± 1.0	3.0 ± 1.0	2.0 ± 0.00	3.3 ± 0.30
Mean	2.88	2.50	3.58	2.00	2.63	2.63	2.38	2.75	2.75	3.13	3.00	2.88	2.50	3.45
Root length (cm)														
0 mM L^{-1}	4.25 ± 0.25	4.0 ± 0.30	8.75 ± 0.20	5.75 ± 0.25	9.5 ± 0.50	5.75 ± 0.25	5.75 ± 0.25	6.0 ± 0.40	4.25 ± 0.25	3.5 ± 0.50	4.3 ± 0.20	3.25 ± 0.25	6.75 ± 0.25	8.0 ± 0.50
1 mM L^{-1}	3.5 ± 0.50	4.75 ± 0.25	9.0 ± 0.25	4.5 ± 0.50	5.75 ± 0.25	5.0 ± 1.0	3.25 ± 0.75	6.3 ± 0.30	3.5 ± 0.50	5.5 ± 0.50	5.5 ± 0.50	4.25 ± 0.25	3.0 ± 1.0	8.3 ± 0.30
4 mM L^{-1}	5.75 ± 0.25	5.0 ± 0.80	9.58 ± 0.20	4.5 ± 0.50	2.0 ± 0.50	4.0 ± 1.0	4.5 ± 0.50	7.6 ± 0.40	8.0 ± 1.0	4.5 ± 0.50	5.45 ± 0.55	2.0 ± 1.0	4.5 ± 0.50	9.0 ± 0.40
10 mM L^{-1}	6.0 ± 0.50	5.5 ± 0.50	9.3 ± 0.40	2.5 ± 0.50	1.5 ± 0.30	4.75 ± 0.75	3.0 ± 1.0	5.8 ± 0.50	4.0 ± 1.0	5.5 ± 0.50	6.0 ± 1.0	2.25 ± 0.75	4.5 ± 0.50	8.8 ± 0.25
Mean	4.88	4.81	9.16	4.31	4.69	4.88	4.13	6.43	4.94	4.75	5.31	2.94	4.69	8.53
Shoot length (cm)														
0 mM L^{-1}	10.0 ± 1.0	7.3 ± 0.30	9.0 ± 0.20	5.75 ± 0.25	7.5 ± 0.50	6.5 ± 0.50	6.25 ± 0.25	8.0 ± 0.30	6.5 ± 0.10	8.0 ± 0.50	6.75 ± 0.75	4.5 ± 0.50	8.0 ± 0.10	9.2 ± 0.30
1 mM L^{-1}	7.0 ± 0.50	7.9 ± 0.40	9.5 ± 0.25	5.75 ± 0.25	7.0 ± 0.50	7.5 ± 0.50	6.5 ± 0.50	9.0 ± 0.40	5.75 ± 0.75	10.0 ± 0.10	6.5 ± 0.30	5.25 ± 0.25	4.25 ± 0.75	9.5 ± 0.40
4 mM L^{-1}	9.5 ± 0.50	8.8 ± 1.0	10.5 ± 0.30	6.0 ± 1.0	4.75 ± 0.75	7.75 ± 0.25	6.75 ± 0.75	8.5 ± 0.40	8.5 ± 0.50	11.0 ± 0.10	6.5 ± 0.20	7.0 ± 0.10	8.5 ± 0.50	10.0 ± 0.30
10 mM L^{-1}	8.75 ± 0.25	10.5 ± 0.50	11.2 ± 0.20	5.5 ± 0.20	5.0 ± 1.0	7.5 ± 0.50	6.0 ± 0.10	9.0 ± 0.50	9.0 ± 0.10	10.25 ± 0.75	8.5 ± 0.50	7.25 ± 0.75	7.5 ± 0.20	10.5 ± 0.25
Mean	8.81	8.63	10.05	5.75	6.06	7.31	6.38	8.63	7.44	9.81	7.06	6.00	7.06	9.80
Root fresh weight (g)														
0 mM L^{-1}	0.011 ± 0.001	0.014 ± 0.001	0.02 ± 0.001	0.004 ± 0.003	0.015 ± 0.003	0.012 ± 0.001	0.008 ± 0.001	0.012 ± 0.002	0.025 ± 0.001	0.004 ± 0.003	0.019 ± 0.005	0.009 ± 0.003	0.007 ± 0.002	0.02 ± 0.001
1 mM L^{-1}	0.01 ± 0.005	0.015 ± 0.003	0.025 ± 0.002	0.005 ± 0.001	0.015 ± 0.003	0.016 ± 0.002	0.005 ± 0.004	0.019 ± 0.003	0.006 ± 0.001	0.012 ± 0.004	0.018 ± 0.004	0.007 ± 0.002	0.006 ± 0.002	0.022 ± 0.003
4 mM L^{-1}	0.013 ± 0.002	0.016 ± 0.001	0.026 ± 0.001	0.008 ± 0.001	0.026 ± 0.002	0.014 ± 0.001	0.014 ± 0.001	0.022 ± 0.002	0.024 ± 0.002	0.023 ± 0.002	0.021 ± 0.001	0.005 ± 0.004	0.011 ± 0.001	0.025 ± 0.001
10 mM L^{-1}	0.012 ± 0.003	0.016 ± 0.006	0.028 ± 0.001	0.009 ± 0.001	0.016 ± 0.004	0.018 ± 0.002	0.005 ± 0.004	0.017 ± 0.003	0.022 ± 0.002	0.019 ± 0.002	0.022 ± 0.002	0.014 ± 0.002	0.015 ± 0.001	0.028 ± 0.002
Mean	0.012	0.015	0.025	0.007	0.018	0.015	0.008	0.018	0.019	0.015	0.020	0.009	0.010	0.024
Shoot fresh weight (g)														
0 mM L^{-1}	0.015 ± 0.001	0.011 ± 0.001	0.018 ± 0.002	0.004 ± 0.001	0.009 ± 0.002	0.011 ± 0.001	0.009 ± 0.001	0.015 ± 0.004	0.052 ± 0.471	0.006 ± 0.003	0.011 ± 0.001	0.005 ± 0.001	0.009 ± 0.001	0.016 ± 0.001
1 mM L^{-1}	0.013 ± 0.001	0.013 ± 0.003	0.02 ± 0.001	0.005 ± 0.001	0.013 ± 0.001	0.013 ± 0.002	0.006 ± 0.003	0.01 ± 0.003	0.006 ± 0.001	0.018 ± 0.006	0.01 ± 0.001	0.005 ± 0.002	0.01 ± 0.001	0.022 ± 0.003
4 mM L^{-1}	0.014 ± 0.002	0.014 ± 0.002	0.015 ± 0.003	0.007 ± 0.002	0.007 ± 0.002	0.012 ± 0.001	0.009 ± 0.002	0.018 ± 0.003	0.023 ± 0.001	0.02 ± 0.005	0.013 ± 0.002	0.009 ± 0.001	0.018 ± 0.003	0.015 ± 0.002
10 mM L^{-1}	0.014 ± 0.007	0.015 ± 0.001	0.018 ± 0.003	0.008 ± 0.001	0.013 ± 0.004	0.015 ± 0.005	0.013 ± 0.002	0.015 ± 0.002	0.02 ± 0.006	0.02 ± 0.002	0.016 ± 0.001	0.012 ± 0.004	0.017 ± 0.002	0.019 ± 0.003
Mean	0.014	0.013	0.018	0.006	0.011	0.013	0.009	0.015	0.025	0.016	0.013	0.008	0.014	0.018
Root dry weight (g)														
0 mM L^{-1}	0.004 ± 0.003	0.004 ± 0.001	0.015 ± 0.002	0.003 ± 0.002	0.009 ± 0.002	0.005 ± 0.003	0.007 ± 0.001	0.008 ± 0.003	0.01 ± 0.001	0.003 ± 0.002	0.01 ± 0.002	0.008 ± 0.003	0.003 ± 0.002	0.012 ± 0.001
1 mM L^{-1}	0.006 ± 0.001	0.005 ± 0.001	0.022 ± 0.001	0.002 ± 0.001	0.005 ± 0.001	0.008 ± 0.002	0.002 ± 0.001	0.01 ± 0.002	0.002 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.004 ± 0.002	0.004 ± 0.001	0.013 ± 0.001
4 mM L^{-1}	0.006 ± 0.002	0.007 ± 0.001	0.025 ± 0.002	0.005 ± 0.001	0.009 ± 0.001	0.006 ± 0.001	0.005 ± 0.001	0.018 ± 0.003	0.007 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.003 ± 0.002	0.005 ± 0.001	0.014 ± 0.002
10 mM L^{-1}	0.009 ± 0.003	0.009 ± 0.006	0.015 ± 0.001	0.004 ± 0.001	0.006 ± 0.001	0.009 ± 0.003	0.002 ± 0.001	0.015 ± 0.002	0.007 ± 0.001	0.006 ± 0.001	0.009 ± 0.001	0.005 ± 0.001	0.004 ± 0.001	0.015 ± 0.001
Mean	0.006	0.006	0.019	0.004	0.007	0.007	0.004	0.013	0.007	0.006	0.008	0.005	0.004	0.014
Shoot dry weight (g)														
0 mM L^{-1}	0.01 ± 0.001	0.009 ± 0.001	0.014 ± 0.001	0.002 ± 0.001	0.008 ± 0.001	0.009 ± 0.002	0.007 ± 0.001	0.006 ± 0.002	0.005 ± 0.002	0.005 ± 0.002	0.007 ± 0.001	0.004 ± 0.001	0.007 ± 0.001	0.013 ± 0.002
1 mM L^{-1}	0.009 ± 0.002	0.007 ± 0.002	0.01 ± 0.002	0.002 ± 0.001	0.006 ± 0.002	0.008 ± 0.001	0.003 ± 0.002	0.006 ± 0.001	0.003 ± 0.002	0.006 ± 0.004	0.009 ± 0.003	0.003 ± 0.001	0.007 ± 0.002	0.009 ± 0.001
4 mM L^{-1}	0.007 ± 0.001	0.007 ± 0.002	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	0.006 ± 0.001	0.007 ± 0.001	0.009 ± 0.002	0.008 ± 0.002	0.008 ± 0.001	0.005 ± 0.002	0.006 ± 0.001	0.007 ± 0.002	0.005 ± 0.002
10 mM L^{-1}	0.008 ± 0.002	0.005 ± 0.001	0.006 ± 0.001	0.004 ± 0.001	0.008 ± 0.001	0.005 ± 0.001	0.008 ± 0.001	0.009 ± 0.003	0.011 ± 0.002	0.012 ± 0.001	0.01 ± 0.002	0.006 ± 0.001	0.008 ± 0.002	0.0055 ± 0.002
Mean	0.009	0.007	0.009	0.003	0.007	0.007	0.006	0.008	0.007	0.008	0.008	0.005	0.007	0.008
Total dry matter (g)														
0 mM L^{-1}	0.014 ± 0.004	0.013 ± 0.001	0.029 ± 0.002	0.005 ± 0.003	0.017 ± 0.003	0.014 ± 0.005	0.014 ± 0.001	0.014 ± 0.003	0.015 ± 0.001	0.008 ± 0.004	0.017 ± 0.001	0.012 ± 0.002	0.01 ± 0.002	0.025 ± 0.001
1 mM L^{-1}	0.015 ± 0.003	0.012 ± 0.003	0.032 ± 0.001	0.004 ± 0.002	0.011 ± 0.001	0.016 ± 0.001	0.005 ± 0.003	0.016 ± 0.002	0.005 ± 0.002	0.013 ± 0.004	0.016 ± 0.002	0.007 ± 0.001	0.011 ± 0.001	0.022 ± 0.002
4 mM L^{-1}	0.013 ± 0.001	0.014 ± 0.001	0.03 ± 0.002	0.01 ± 0.001	0.014 ± 0.001	0.012 ± 0.002	0.012 ± 0.002	0.027 ± 0.001	0.015 ± 0.001	0.015 ± 0.002	0.012 ± 0.001	0.009 ± 0.002	0.012 ± 0.001	0.019 ± 0.002
10 mM L^{-1}	0.017 ± 0.005	0.014 ± 0.007	0.021 ± 0.002	0.008 ± 0.002	0.014 ± 0.002	0.014 ± 0.002	0.01 ± 0.002	0.024 ± 0.001	0.018 ± 0.002	0.018 ± 0.002	0.019 ± 0.003	0.011 ± 0.001	0.012 ± 0.001	0.020 ± 0.001
Mean	0.015	0.013	0.028	0.007	0.014	0.014	0.010	0.020	0.013	0.014	0.016	0.010	0.011	0.022
Nitrogen efficiency (%)														
4 mM L^{-1}	128.57	100.00	200.00	40.00	120.00	133.33	42.86	66.67	37.50	75.00	180.00	50.00	100.00	180.00
10 mM L^{-1}	112.50	140.00	166.67	50.00	75.00	160.00	37.50	66.67	27.27	50.00	90.00	50.00	87.50	163.64

Continued

N treatment	BCW-56	RPHR-1005	EPLT-109	JAYA	JGL-MASURI	JGL-1798	SWARNA	MTU 1010	BPT 5204	BADRAKALI	ERRAMALLELU	IMPROVED SAMBA MASURI	
Total number of leaves													
0 mM L^{-1}	3.0 ± 1.0	2.5 ± 0.50	2.5 ± 0.50	2.5 ± 0.50	2.0 ± 0.00	3.0 ± 1.0	2.5 ± 0.50	3.0 ± 1.0	1.5 ± 0.50	2.5 ± 0.50	2.5 ± 0.50	3.0 ± 0.00	
1 mM L^{-1}	2.5 ± 0.50	2.5 ± 0.50	2.0 ± 0.00	2.0 ± 0.00	2.5 ± 0.50	2.0 ± 0.00	3.0 ± 1.0	2.0 ± 0.00	2.0 ± 0.40	2.0 ± 0.00	2.5 ± 0.50	3.0 ± 1.0	
4 mM L^{-1}	3.0 ± 0.00	2.0 ± 0.00	2.5 ± 0.50	3.0 ± 0.00	2.5 ± 0.50	2.0 ± 0.00	2.0 ± 0.00	2.5 ± 0.50	2.3 ± 0.50	2.5 ± 0.50	2.5 ± 0.50	3.0 ± 1.0	
10 mM L^{-1}	3.0 ± 1.0	2.5 ± 0.50	2.5 ± 0.50	3.0 ± 1.0	3.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00	3.0 ± 0.00	2.0 ± 1.0	2.5 ± 0.50	2.0 ± 0.00	2.0 ± 0.00	
Mean	2.88	2.38	2.38	2.63	2.50	2.25	2.38	2.63	1.95	2.38	2.38	2.75	
Root length (cm)													
0 mM L^{-1}	7.25 ± 0.25	4.25 ± 0.25	6.0 ± 1.0	6.75 ± 0.25	3.25 ± 0.25	9.25 ± 0.75	3.0 ± 0.50	9.25 ± 1.0	2.2 ± 0.40	6.0 ± 1.0	4.25 ± 0.25	4.75 ± 0.75	
1 mM L^{-1}	3.25 ± 0.25	3.5 ± 0.50	3.0 ± 1.0	4.0 ± 1.0	7.5 ± 0.50	3.0 ± 0.50	4.0 ± 1.0	7.0 ± 0.50	3.0 ± 0.30	3.0 ± 0.50	2.25 ± 0.25	4.25 ± 0.75	
4 mM L^{-1}	3.25 ± 0.75	3.5 ± 0.50	4.5 ± 0.50	4.0 ± 1.0	7.75 ± 0.75	3.75 ± 0.75	4.5 ± 0.50	3.0 ± 1.0	2.0 ± 1.0	3.25 ± 0.25	3.5 ± 0.50	4.5 ± 0.50	
10 mM L^{-1}	6.25 ± 0.75	3.5 ± 0.50	3.75 ± 0.25	3.5 ± 0.50	3.5 ± 0.50	4.5 ± 0.50	6.0 ± 1.0	3.0 ± 1.0	1.75 ± 0.10	4.5 ± 0.50	3.0 ± 1.0	3.25 ± 0.25	
Mean	5.00	3.69	4.31	4.56	5.50	5.13	4.38	5.56	2.24	4.19	3.25	4.19	
Shoot length (cm)													
0 mM L^{-1}	9.75 ± 0.25	8.75 ± 0.75	9.75 ± 0.25	6.75 ± 0.25	5.5 ± 0.50	6.0 ± 0.10	6.65 ± 0.65	5.5 ± 0.50	2.9 ± 0.50	8.0 ± 0.30	7.75 ± 0.25	5.75 ± 0.05	
1 mM L^{-1}	7.5 ± 0.30	7.75 ± 0.25	9.75 ± 0.25	9.25 ± 0.05	8.75 ± 0.45	5.75 ± 0.25	6.85 ± 0.85	7.25 ± 0.25	4.0 ± 0.75	9.0 ± 0.20	7.75 ± 0.75	9.25 ± 0.75	
4 mM L^{-1}	10.5 ± 0.50	8.0 ± 0.10	9.0 ± 0.10	7.25 ± 0.15	8.25 ± 0.25	7.5 ± 0.50	7.75 ± 0.75	6.5 ± 0.30	4.2 ± 0.50	8.0 ± 0.50	9.5 ± 0.50	8.0 ± 0.20	
10 mM L^{-1}	9.0 ± 0.10	7.25 ± 0.25	9.5 ± 0.50	9.0 ± 0.50	13.0 ± 0.20	6.5 ± 0.70	8.75 ± 0.25	9.5 ± 0.20	3.6 ± 0.40	11.0 ± 0.10	10.0 ± 0.20	5.25 ± 0.25	
Mean	9.19	7.94	9.50	8.06	8.88	6.44	7.50	7.19	3.68	9.00	8.75	7.06	
Root fresh weight (g)													
0 mM L^{-1}	0.021 ± 0.001	0.01 ± 0.006	0.023 ± 0.003	0.019 ± 0.001	0.005 ± 0.001	0.021 ± 0.002	0.011 ± 0.001	0.027 ± 0.004	0.004 ± 0.004	0.022 ± 0.001	0.02 ± 0.003	0.015 ± 0.001	
1 mM L^{-1}	0.022 ± 0.004	0.009 ± 0.003	0.011 ± 0.001	0.021 ± 0.009	0.015 ± 0.01	0.007 ± 0.001	0.014 ± 0.001	0.023 ± 0.003	0.005 ± 0.005	0.01 ± 0.001	0.012 ± 0.001	0.009 ± 0.002	
4 mM L^{-1}	0.024 ± 0.002	0.007 ± 0.001	0.017 ± 0.001	0.016 ± 0.015	0.007 ± 0.001	0.009 ± 0.001	0.018 ± 0.001	0.015 ± 0.002	0.006 ± 0.009	0.009 ± 0.008	0.018 ± 0.007	0.025 ± 0.005	
10 mM L^{-1}	0.025 ± 0.003	0.012 ± 0.001	0.007 ± 0.006	0.026 ± 0.002	0.013 ± 0.009	0.013 ± 0.005	0.02 ± 0.006	0.023 ± 0.002	0.02 ± 0.006	0.016 ± 0.002	0.011 ± 0.003	0.01 ± 0.001	
Mean	0.023	0.010	0.015	0.021	0.010	0.013	0.016	0.022	0.009	0.014	0.015	0.015	
Shoot fresh weight (g)													
0 mM L^{-1}	0.016 ± 0.001	0.01 ± 0.005	0.017 ± 0.001	0.016 ± 0.001	0.008 ± 0.001	0.014 ± 0.001	0.011 ± 0.001	0.012 ± 0.002	0.006 ± 0.003	0.01 ± 0.001	0.016 ± 0.001	0.013 ± 0.001	
1 mM L^{-1}	0.01 ± 0.002	0.01 ± 0.003	0.012 ± 0.002	0.013 ± 0.002	0.015 ± 0.002	0.007 ± 0.001	0.012 ± 0.002	0.01 ± 0.005	0.005 ± 0.001	0.01 ± 0.001	0.009 ± 0.004	0.012 ± 0.001	
4 mM L^{-1}	0.026 ± 0.009	0.011 ± 0.005	0.016 ± 0.006	0.022 ± 0.008	0.011 ± 0.001	0.016 ± 0.001	0.013 ± 0.004	0.015 ± 0.012	0.012 ± 0.004	0.005 ± 0.003	0.023 ± 0.008	0.03 ± 0.01	
10 mM L^{-1}	0.021 ± 0.003	0.016 ± 0.003	0.017 ± 0.001	0.015 ± 0.003	0.02 ± 0.002	0.012 ± 0.001	0.012 ± 0.003	0.02 ± 0.003	0.015 ± 0.003	0.012 ± 0.002	0.01 ± 0.009	0.011 ± 0.003	
Mean	0.018	0.012	0.016	0.017	0.014	0.012	0.012	0.014	0.010	0.009	0.015	0.017	
Root dry weight (g)													
0 mM L^{-1}	0.013 ± 0.003	0.006 ± 0.004	0.01 ± 0.001	0.016 ± 0.001	0.004 ± 0.001	0.007 ± 0.002	0.005 ± 0.002	0.011 ± 0.001	0.003 ± 0.001	0.009 ± 0.001	0.006 ± 0.001	0.004 ± 0.001	
1 mM L^{-1}	0.01 ± 0.001	0.003 ± 0.001	0.004 ± 0.002	0.009 ± 0.001	0.005 ± 0.002	0.006 ± 0.001	0.005 ± 0.001	0.007 ± 0.001	0.002 ± 0.001	0.006 ± 0.001	0.005 ± 0.002	0.006 ± 0.001	
4 mM L^{-1}	0.01 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.005	0.003 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	0.009 ± 0.001	0.003 ± 0.002	0.006 ± 0.002	0.003 ± 0.002	0.008 ± 0.003	
10 mM L^{-1}	0.008 ± 0.001	0.003 ± 0.001	0.003 ± 0.002	0.008 ± 0.001	0.005 ± 0.004	0.006 ± 0.001	0.006 ± 0.002	0.008 ± 0.001	0.002± 0.001	0.007 ± 0.001	0.008 ± 0.001	0.005 ± 0.001	
Mean	0.010	0.005	0.006	0.010	0.004	0.006	0.005	0.009	0.003	0.007	0.006	0.006	
Shoot dry weight (g)													
0 mM L^{-1}	0.009 ± 0.001	0.007 ± 0.002	0.009 ± 0.001	0.012 ± 0.002	0.006 ± 0.001	0.011 ± 0.002	0.01 ± 0.001	0.006 ± 0.001	0.002 ± 0.001	0.008 ± 0.001	0.006 ± 0.002	0.004 ± 0.003	
1 mM L^{-1}	0.006 ± 0.001	0.004 ± 0.001	0.002 ± 0.001	0.011 ± 0.003	0.007 ± 0.001	0.003 ± 0.002	0.007 ± 0.001	0.006 ± 0.002	0.002 ± 0.002	0.006 ± 0.001	0.005 ± 0.002	0.005 ± 0.001	
4 mM L^{-1}	0.009 ± 0.003	0.006 ± 0.002	0.008 ± 0.001	0.015 ± 0.005	0.004 ± 0.003	0.009 ± 0.004	0.007 ± 0.001	0.005 ± 0.001	0.009 ± 0.004	0.006 ± 0.004	0.008 ± 0.002	0.01 ± 0.005	
10 mM L^{-1}	0.009 ± 0.001	0.006 ± 0.001	0.01 ± 0.005	0.011 ± 0.002	0.011 ± 0.003	0.006 ± 0.002	0.006 ± 0.001	0.01 ± 0.002	0.01 ± 0.001	0.01 ± 0.001	0.007 ± 0.006	0.006 ± 0.001	
Mean	0.008	0.006	0.007	0.012	0.007	0.007	0.008	0.007	0.006	0.008	0.007	0.006	
Total dry matter (g)													
0 mM L^{-1}	0.022 ± 0.002	0.013 ± 0.005	0.019 ± 0.002	0.028 ± 0.001	0.01 ± 0.001	0.018 ± 0.003	0.015 ± 0.003	0.017 ± 0.002	0.005 ± 0.003	0.017 ± 0.002	0.012 ± 0.002	0.008 ± 0.002	
1 mM L^{-1}	0.016 ± 0.001	0.007 ± 0.002	0.006 ± 0.001	0.02 ± 0.002	0.012 ± 0.003	0.009 ± 0.002	0.012 ± 0.002	0.013 ± 0.003	0.004 ± 0.002	0.012 ± 0.001	0.01 ± 0.003	0.011 ± 0.001	
4 mM L^{-1}	0.019 ± 0.004	0.012 ± 0.002	0.014 ± 0.002	0.021 ± 0.010	0.007 ± 0.004	0.014 ± 0.004	0.012 ± 0.002	0.014 ± 0.001	0.012 ± 0.003	0.012 ± 0.002	0.011 ± 0.004	0.018 ± 0.007	
10 mM L^{-1}	0.017 ± 0.002	0.009 ± 0.002	0.013 ± 0.007	0.019 ± 0.002	0.016 ± 0.006	0.012 ± 0.003	0.012 ± 0.001	0.018 ± 0.003	0.012 ± 0.004	0.017 ± 0.002	0.015 ± 0.007	0.011 ± 0.001	
Mean	0.019	0.010	0.013	0.022	0.011	0.013	0.013	0.016	0.008	0.015	0.012	0.012	
Nitrogen efficiency (%)													
4 mM L^{-1}	66.67	66.67	25.00	73.33	175.00	33.33	100.00	120.00	22.22	100.00	62.50	50.00	
10 mM L^{-1}	66.67	66.67	20.00	100.00	63.64	50.00	116.67	60.00	20.00	60.00	71.43	83.33	

The data are mean ± standard error (n = 2)

Table 2 (a). Correlation matrix for various growth dynamic traits under nitrogen deficient condition.

	TNL/P	RL(Cm)	SL(Cm)	RFW(g)	SFW(g)	RDW(g)	SDW(g)	TDM(g)
TNL/P	1							
RL(Cm)	.610**	1						
SL(Cm)	.292	.424*	1					
RFW(g)	.292	.564**	.422*	1				
SFW(g)	-.028	.017	.190	.576**	1			
RDW(g)	.376	.553**	.414*	.698**	.381	1		
SDW(g)	.392*	.603**	.675**	.542**	.184	.708**	1	
TDM(g)	.415*	.624**	.580**	.676**	.312	.933**	.914**	1

* and ** Significant at 5% and 1% levels.

Table 2(b). Correlation matrix for various growth dynamic traits under low nitrogen condition.

	TNL/P	RL(Cm)	SL(Cm)	RFW(g)	SFW(g)	RDW(g)	SDW(g)	TDM(g)
TNL/P	1							
RL(Cm)	.537**	1						
SL(Cm)	.478*	.450*	1					
RFW(g)	.407*	.642**	.518**	1				
SFW(g)	.684**	.603**	.649**	.632**	1			
RDW(g)	.572**	.584**	.456*	.759**	.599**	1		
SDW(g)	.436*	.415*	.400*	.597**	.745**	.585**	1	
TDM(g)	.579**	.578**	.485*	.777**	.733**	.935**	.834**	1

* and ** Significant at 5% and 1% levels.

Table 2(c). Correlation matrix for various growth dynamic and nitrogen efficiency traits under moderate nitrogen condition.

	TNL/P	RL(Cm)	SL(Cm)	RFW(g)	SFW(g)	RDW(g)	SDW(g)	TDM(g)	NE (%)
TNL/P	1								
RL(Cm)	.673**	1							
SL(Cm)	.667**	.568**	1						
RFW(g)	.473*	.400*	.405*	1					
SFW(g)	.392*	.175	.439*	.466*	1				
RDW(g)	.709**	.677**	.449*	.643**	.122	1			
SDW(g)	.103	-.166	.023	.057	.611**	-.153	1		
TDM(g)	.736**	.596**	.449*	.652**	.365	.917**	.253	1	
NE (%)	.381	.457*	.275	.350	-.212	.499**	-.545**	.269	1

Table 2(d). Correlation matrix for various growth dynamic and nitrogen efficiency traits under high nitrogen condition.

	TNL/P	RL(Cm)	SL(Cm)	RFW(g)	SFW(g)	RDW(g)	SDW(g)	TDM(g)	NE (%)
TNL/P	1								
RL(Cm)	.467*	1							
SL(Cm)	.565**	.452*	1						
RFW(g)	.648**	.520**	.275	1					
SFW(g)	.631**	.464*	.560**	.577**	1				
RDW(g)	.582**	.772**	.457*	.616**	.326	1			
SDW(g)	.370	-.046	.497**	.335	.599**	.019	1		
TDM(g)	.683**	.626**	.645**	.694**	.588**	.853**	.539**	1	
NE (%)	.257	.526**	.069	.332	-.056	.491*	-.486*	.160	1

In the present investigation, we assessed twenty six rice genotypes for their growth dynamics and nitrogen efficiency with respect to different nitrogen treatment levels *viz.,* nitrogen deficiency (0 mM L^{-1})/T1, low nitrogen (1 mM L^{-1})/ T2, medium nitrogen (4 mM L^{-1})/T3/ and high nitrogen (10 mM L^{-1})/T4 treatments.

Our findings indicated that there was a high genotypic variation among genotypes for growth dynamics traits viz., morphological (TNL, RL and SL) and biomass (RFW, SFW, RDW, SDW and TDM) different nitrogen levels. High growth performance was observed in DRRH3 and low in BPT5204. As reported in previous studies the concentration of nitrogen sternly affects the total number of leaves, in DRRH3 total number of leaves increased with the concentration of nitrogen, however at higher concentration the number of leaves declined slowly. It is attributed that nitrogen concentration modulates the hormone gibberellins indirectly through cyctokinins and enhance the number of leaves per plant (10) (23) (38). Maximum total dry matter accumulation recorded in the DRRH3 and minimum dry matter accumulation was observed in B-95-1 and high genotypic variation was observed among the genotypes. However, as reported earlier (14) not much variation was observed within the genotype among four treatments. Root trait is an important one which is influenced by both genetically and surrounding environments. In the present study root length significantly influenced nitrogen concentration. Most of the genotypes recorded greater root lengths at nitrogen deficient conditions and low nitrogen conditions. In contrast, most of genotypes displayed low growth rates at high nitrogen concentrations. This is probably at low nitrogen conditions roots penetrate into deep soil layers for nutrients absorption.

Root is an essential plant organ which absorbs water and nutrients from soil and plays a significant role in growth and development of plants. The concentration of N significantly affects the root growth and its functional ability. Rice genotypes significantly exhibited variations in shoot-root ratio; root length and root dry weight for N treatments. Rice genotypes such as DRRH3 recorded higher biomass in their roots at low N levels as well at high N levels. High nitrogen genotypes recorded higher biomass than low nitrogen efficient genotypes of rice (17). The reason for the accumulation high biomass in the high efficient genotypes could be the effective utilization of accumulated N in protein synthesis (17). In the present study, nitrogen concentration increases the biomass of the roots. As reported earlier nitrogen treatment level significantly improved the root growth in terms of root dry weight of upland rice genotypes (13). The increase of root dry weight

significantly enhanced yield in upland rice genotypes by improving water and nutrient efficiency (13). The plant growth is regulated by supply of carbon from shoot to root via phloem transport as well as nitrogen from root to shoot via xylem transport (35). The fluxes are possibly depends on the concentration gradients of carbon, nitrogen, mineral nutrients in the shoot, roots and various environmental factors (41). According to Cooper and Clarkson (1989)(9) the increase of carbon transportation and root-shoot dry weight under N limited condition is the important aspect to improve the nitrogen efficiency in the genotypes.

Based on NE data in the present study of 26 genotypes were categorized into high nitrogen efficient (HNE) genotypes *viz.,* KMR3R, RPHR-196, DRRH3, NLR-3042, SC5-2-2-1, KRH2 and SWARNA, moderate nitrogen efficient (MNE) genotypes *viz.,* RPHR-111-3, BI33, RAMAPPA, BCW-56, RPHR-1005, JAYA, JGL-MAHSURI, MTU1010, BADRAKALI, ERRAMALLELU and IMPROVED SAMBA MAHSURI and low nitrogen efficient (LNE) genotypes *viz.,* B-95-1, AJAYA-R, EPLT-104, DR714-1-2R, WGL-347, EPLT-109, JGL-1798 and BPT5204 at both N treatment (4 mM L^{-1} and 10 mM L^{-1}) levels. The high NE genotypes probably efficiently utilize the accumulated nitrogen in protein synthesis resulted in high biomass accumulation. Our data suggested that at low soil nitrogen conditions high nitrogen efficient genotypes (HNE) showed higher nitrogen efficiency than medium (MNE) and low (LNE) nitrogen efficient genotypes. Therefore HNE genotypes were highly desirable at low soil nitrogen levels (4).

Further, statistical significance was done by multivariate statistical analysis which gave a clear, confident and accurate analysis on association of multiple complex traits of genotypes at different N treatments. Multivariate analysis is a widely used technique for identification, separation of genotypes and germplasm based on morphological, biochemical or molecular markers (22). Multivariate analysis includes Pearson correlation, principal component analysis, hierarchical heatmap and clustering analysis (24).

In the present Pearson correlation analysis indicated that rice root and shoot traits are major contributed traits for tolerant to different nitrogen regimes. Root and shoot organs were directly involved in acquisition and assimilation of nitrogen in different parts of the plants. Directly and indirectly contributed traits would more useful for selection process and definitely helpful to the breeders to develop better genotypes (29).

PCA is one of the multivariate statistical technique for simplify complex data sets (7) (12). PCA reduces the “n” variables to “r” new variables in a given “m” observations (30). The PCA identified most contributed traits and their variability with response to nitrogen treatment levels among genotypes. In the present study high variability was observed in the range of 88-90% among the measured traits such as growth dynamics and nitrogen efficiency traits at different nitrogen treatment levels. Based on this variability it was concluded that DRRH3 as nitrogen tolerant and BPT5204 as nitrogen sensitive genotypes among 26 genotypes at various nitrogen regimes. PCA data also revealed that most contributed traits for genotypic variation were root, shoot dynamics, leaves number and dry matter. The PCA data is in agreement with correlation studies. Earlier findings also reported that the high level variability among genotypes and measured traits resulted in high level of positive effect, which is further useful for improvement of genotypes during breeding programme (15) (3).

Hierarchical clustering of heatmap is a graphical representation and visual method that can be used to observe the complex associations of multiple traits and variations in the genotypes irrespective of their treatment levels. The heatmap and hierarchical clustering was measured with hierarchy based on the distance or similarity way (21). In the present study genotypes and traits measured traits were clustered into different groups, from which it was concluded that the DRRH3 is a nitrogen tolerant genotype where as BPT5204 is a sensitive genotype. Measured traits such as growth dynamics and nitrogen efficiency traits are more responsible traits for the genotypic

variation and these traits were grouped into three groups such as group I, group II, and group III at each treatment levels.

Conclusion

In conclusion of our data, based on growth dynamics, biomass as well as nitrogen efficiency traits confirmed DRRH3 as high nitrogen efficient genotype and BPT5204 as low nitrogen efficient genotype among the genotypes studied at various nitrogen levels. These results were further strongly supported by multivariate statistical analysis. The root and shoot parameters were significantly positively correlated with nitrogen efficiency trait at different N levels. These root and shoot parameters were significantly influenced by genotypes as well as N treatments. Hence, while developing NUE rice genotypes breeders should focus on these traits using adequate N rate.

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Antidepressant-like effects of methanolic extract of *Xanthium strumarium* (*Asteraceae*) in mice

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Abstract

Plants and other natural substances have been used as the rich source of medicine. *Xanthium strumarium* plant is reported in ethno-pharmacological study as medicinal plant. Several phytochemical and pharmacological experiments already done of *X. strumarium*. However, there are no scientific reports about the antidepressant-like activity of *X. strumarium*. The aim of study is evaluation the antidepressant-like activity of methanolic extract of *Xanthium strumarium* (MEXS).The antidepressant-like activity evaluated by Tail Suspension Test (TST). Administration of MEXS extract of 50, 100, and 200mg/kg significantly ($p< 0.001$) decreased the immobility periods of mice when compared to the control group (0.9% normal saline water), indicating significant antidepressant-like activity. The positive control imipramine hydrochloride (30mg/kg) also showed similar effect as MEXS.The experimental data clearly demonstrate that the methanolic extract of *X. strumarium* possesses antidepressant-like activity in the animal model.

Keywords: *Xanthium strumarium*;Ethno-pharmacological; Tail Suspension Test; Antidepressant; Imipramine hydrochloride

Introduction

Plants produce a diverse range of bioactive compounds as a rich source of different types of medicines. Higher plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care since ancient period (1). *Xanthium strumarium* L. is a cocklebur or burweed commonly found as a weed in roadsides, rice fields, hedges throughout the tropical parts of Bangladesh and India subcontinent (2). The genus *Xanthium* (Family: *Asteraceae*) is imparted by 25 species, amongst them three species and one variety found in Bangladesh (3). The word "Xanthium" derived from an ancient Greek word "Xanthos" meaning yellow and "strumarium" means "cushionlike swelling," which turn from green to yellow as they ripen (later they become deep yellow to brown) (4). Alternate triangular-ovate or suborbicular leaves, light and bright green in color in an alternate pattern with irregular lobes as well as relatively inconspicuous teeth such as 5–15 cm long, often three-lobed, prominent veins, long petiole, scabrous on both sides.

X.strumarium, used as renowned herbal medicines in China, Europe, Indo-China, Malaysia and America(5).Chinese people used *X. strumarium* fruits for the treatment of different kind inflammatory diseases including bronchitis, chronic rhinitis, allergic rhinitis, lumbago, tympanitis, urticaria and arthritis,ozena and other ailments(6). It has also reported that *X. strumarium* used as a medicine for curing nasal sinusitis, vomiting, and headache (7).Various Native American tribes used *X. strumarium* to relieve constipation and diarrhea (8). In southern part of Bangladesh, people used *X. strumarium* for the treatment of several ailments including asthma, diabetes mellitus, jaundices, gastritis (upper abdominal discomfort), urinary disorders and as blood purifier(9).

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X. strumarium leaves have reported to contains alkaloids, flavonoids (flavonol), (10) anthraquinone, cardenolide, leucoanthocyanin, simple phenolics (Catechol) and triterpenoids(11). Several studies have reported *X. strumarium* include phenolic compounds as thiazolidinediones, chlorogenic acids, ferulic acids (12), 1,3,5-tri-*O*-caffeoyl quinic acid, 1,5-di-*O*caffeoyl quinic acid, caffeic acid (13), as well as isoprenoids such as stigmasterol, â-sitosterol(14), monoterpene and sesquiterpene hydrocarbons (15), triterpenoid saponins(16).

Earlier study reported that *X. strumarium* has significant anti-inflammatory and analgesic properties in mice [17]. The whole plant used to treat cytotoxicity and antitumor activity [18]. Furthermore, several investigation has reported that *X. strumarium* possesses anti-ulcerogenic [19], anthelmintic [20], diuretic [21], antimicrobial, antioxidant [22], and antilipidemic activity [23]. *X. strumarium* traditionally used central nervous system (CNS) stimulant agent which may have potential antidepressant activity. Our current study designed to confirm the anti-depressant effects of *X. strumarium*in mice model.

Methods

Plant material and extraction : The fresh plant collected from the area of Narail district during the month of April, 2016. The *X. strumarium* was taxonomically identified by Chief Scientist and Taxonomist of Bangladesh. The leaves parts of the plant *Xanthium strumarium* washed with water to remove adhering dirt and then cut into small pieces, dried for 4 days and finally dried at 45ºC for 36h in an electric oven. After complete drying, the entire portions were pulverized into a coarse powder with help of a grinding machine and stored in an airtight container for further use. About 400g of powdered material soaked in methanol in a beaker at 25±2°C and mixture need to be stirred using a sterile glass rod. The solution filtered through a cloth; the marc strained through a special press. The crude extract used for the investigation of the antidepressant-like effect of the methanolic extract of *X. strumarium* in mice model.

Animals : Swiss albino mice selected for the recent experiment at the age of 3-4 weeks, weighing between 20-25g collected from Jahangirnagar University. Animals maintained under standard environmental conditions (temperature: (23±2ºC), relative humidity: 55-65% and 12h light/12h dark cycle). The animals acclimatized to laboratory condition for one week before the experiment. All protocols for the animal experiment approved by the institutional animal ethics committee, Jessore University of Science And Technology. Animals (1995) formulated by The Swiss Academy of Medical Sciences and the Swiss Academy of Sciences. All experimental rules approved by the Institutional Animal Ethical Committee of Jessore University of Science & Technology.

Drugs and treatments : The investigation of pharmacological activity of X. strumarium we used chemicals and drugs such as methanol (Merck, Germany), Imipramine hydrochloride (Square Pharmaceutical Ltd, Bangladesh) and 0.9% sterile normal saline solution (Beximco Infusion Ltd). The standard drug Imipramine hydrochloride (30mg/kg) used in antidepressant activity test. The MEXS (50, 100, and 200mg/kg) dissolved in Dimethyl sulfoxide (DMSO) whereas, the standard drug Imipramine hydrochloride (30mg/kg) prepared by 0.9% normal saline water.The test and standard groups received MEXS and drugs orally 30 min before the experiments. On the other hand, the control group received 0.1mL/mouse 0.9% normal saline water. All the groups received drugs and samples via gavage. All the chemicals and the drugs were analytical graded and highly purified.

Anti-depressant activity test

Tail suspension test : The tail suspension test (TST) conducted as initially described by *Steru et al.* (1985) with modifications (17). One hour after oral administration and 30min after intraperitoneal injection of test compounds, mice individually suspended by the tail from a horizontal ring-stand bar raised 30 cm above the floor using adhesive tape placed 1 cm from the tip of tail and positioned such that the base of their tail was aligned with the horizontal plane. Test sessions

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lasted for 6min. Behaviors for the last 4 of the 6min period were then analyzed. Immobility was measured, a mouse judged to be immobile when it hung by its tail without engaging in any active behavior.

Statistical analysis : The results are presented as mean±SEM. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test as appropriate using SPSS 20 software. Differences between groups considered significant at a level of $p< 0.001$.

Result and discussion

The tail suspension test investigated anti-depressant activity of methanolic extract of *Xanthium strumarium* at the doses of 50,100 and 200 mg/kg body weight. TST induced immobility is reduced by a large number of clinically active and atypical antidepressant effect. The antidepressant activity of tail suspension test significantly ($p< 0.001$) increased at the dose of 50 mg/kg and 100mg/kg body weight mostly decreases the immobility time on test animals.MEXS at 200 mg/kg dose also observed decreases the immobility time of the test animals. The mean values of MEXS (50, 100, and 200mg/kg) compare to the negative control group. Dunnett's post hoc analysis demonstrated that the test treatments significantly decreased the duration immobility in comparison to the control group ($p< 0.001$). Likewise, the extract reduced the duration of immobility time in the tail suspension test (Table 1 and Figure. 1). Post hoc analysis confirmed that the extract significantly decreased the immobility time in comparison to the control group ($p< 0.001$).

The aims of this study assessed the antidepressant-like effect of MEXS using animal behavioral models.Our present study indicates that the antidepressant-like effect of MEXS found to comparable with the standard drug Imipramine hydrochloride (30mg/kg).Imipramine hydrochloride acts by inhibiting norepinephrine reuptake and has used as a standard drug in majority studies(18). The beneficial effects of Imipramine hydrochloride in TST model seems to be due to increased availability of these neurotransmitters (NE) and serotonin (5HT) at the postsynaptic site following reuptake inhibition(19). Some studies already have shown the adaptogenic effects of the plant extract via normalization of the various stress parameters and monoaminergic levels which may provide a hint that the extract is bringing their possible antidepressant-like effect through the restoration of normal monoaminergic NE(20). The action of the triterpenoid and saponins resulted in the enhancements of the nerve impulse transmission (21). Neurochemical assays suggested that treatment by triterpenoid and saponins improved brain antioxidant activity to varying degrees after the behavioral despair test(22).The pattern of CNS effects observed through this experiment suggests us the involvement of norepinephrine NE system on its antidepressant-like effect.

Conclusion

In conclusion, the effects of MEXS may possess antidepressant-like effect in the classical

Table1: Anti-depressant activity of MEXS on tail suspension test in mice.

Treatment	Dose	Duration of Immobility (seconds)
Control (saline water)	0.1ml/mouse	84±2.70
Imipramine	30 mg/kg	574±1.67*
MEXS	50mg/kg	560±1.58*
MEXS	100mg/kg	484±1.04*
MEXS	200mg/kg	404±1.76*

Each value represented as the mean±SEM (n=5), MEXS = Methanolic extract of *X. strumarium* leaves. *$p< 0.001$ compared with the control group (Dunnett's Test).

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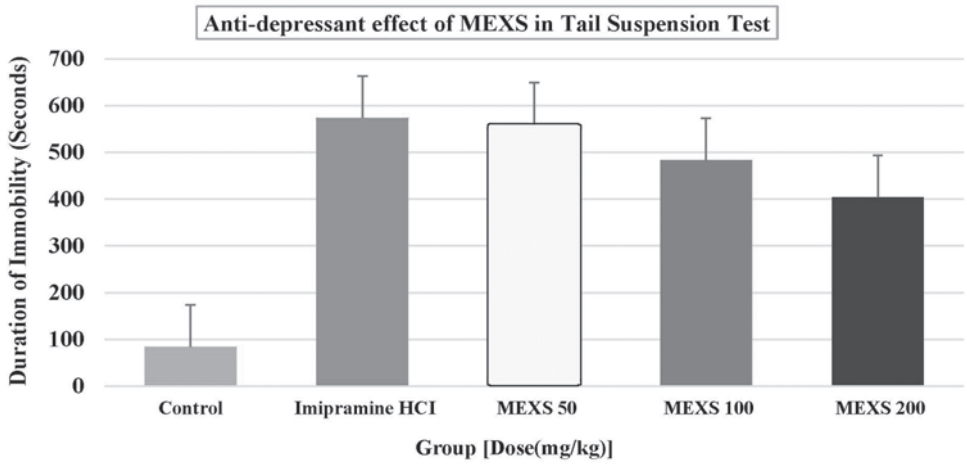

Fig. 1: Anti-depressant activity of MEXS on tail suspension test (duration of Immobility in second) in mice.

model like TST comparable to the standard drug Imipramine hydrochloride.However, further study must need to elucidate the mechanism of action of MEXS in the CNS, the pattern of effects observed in this experiment suggest the involvement of the norepinephrine neurotransmitters system on its antidepressant-like effect.Further investigation needs to identify and isolate the active compounds in *X. strumarium.*

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Authors' contributions : Md. Rashidur Rahman designed and coordinated all laboratory experiments, analyzed and interpreted results. Sheikh Nasrin Keya conducted all experiments. Md. Shahed-Al-Mahmud done statistical analysis and drafted the manuscript. All authors read and approved the manuscript.

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Polysorbate 80 Interferes in the Extrinsic Fluorescence based Tertiary Structure Determination of a Therapeutic Anti-CD20 Antibody with ANS Dye

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Abstract

Therapeutic proteins such as monoclonal antibodies are complex molecules, need extensive characterization before their release into the market for clinical use. Therapeutic protein formulations are routinely characterized for their tertiary structure. Extrinsic fluorescence spectroscopy using various molecular rotors such as polarity sensitive ANS dyes is commonly used for the tertiary structure determination. However, the formulation excipient profile containing surfactants such polysorbate is known to have profound influence in terms of background noise in the extrinsic fluorescence when ANS dyes are used. However, there is neither any study that has clearly indicated the interference of polysorbate 80 in the extrinsic fluorescence profile of a formulated IgG1 monoclonal antibody drug product nor any study has reported the solution to the problem of avoiding interference from polysorbate 80 during extrinsic fluorescence analysis of a formulated monoclonal antibody drug product. Here in this study we reported the interference of polysorbate 80 in the extrinsic fluorescence profile of therapeutic protein drug product and recommending a polysorbate 80 removal step prior to extrinsic fluorescence analysis of drug product for the tertiary structure determination.

Keywords: Polysorbate, extrinsic fluorescence, anti-CD20 antibody, tertiary structure, ANS dye

Introduction

Biopharmaceuticals are complex proteins and are susceptible to degradations. Even minor changes in solution chemistry affects the degradation profile of therapeutic proteins such as monoclonal antibodies. The conformational and compositional stability of therapeutic proteins exists only within a relatively narrow range of osmolality and pH, and mostly excipients are also required to be added to increase their stability (1). Extensive physico chemical characterization of proteins is a prerequisite for the development of stable formulations and their clinical use as per regulatory guidelines (2, 3). Fluorescence spectroscopy is highly sensitive method often employed for structural characterization of recombinant therapeutic proteins and monoclonal antibodies. Stable formulations of therapeutic IgG1 antibody are subjected for Intrinsic and extrinsic protein fluorescence using appropriate dyes for the determination of their secondary and tertiary structure respectively.

Different varieties of molecular rotors are routinely employed for the determination of extrinsic fluorescence to analyze the stressed drug product samples for aggregates and to analyze their tertiary structure (4). ANS (8-Anilinonaphthalene-1-sulfonic acid) dye is one of the commonly used polarity responsive fluorescent dye for the extrinsic fluorescence analysis for the determination of tertiary structure.

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Extrinsic dyes can be covalently attached to proteins, e.g. via the [-amino group of lysine, the α-amino group of the N-terminus, or the thiol group of cysteine. More interesting for the analysis of pharmaceutical formulations are extrinsic dyes that interact non-covalently with proteins and protein degradation products, through hydrophobic or electrostatic interactions.

The fluorescence properties of ANS and other polarity sensitive dyes strongly depend on their interaction with protein molecules, which results in changes of polarity and viscosity of the environment. Hydrophobic interactions and electrostatic interactions have been discussed as binding mechanisms of ANS to proteins (5). However, previous research reported that most polarity responsive dyes are interfered with the extrinsic fluorescence of polysorbate containing protein formulations via dye-surfactant interactions and give high background fluorescence (4).Almost all the stable formulations of biopharmaceuticals especially monoclonal antibody formulations contain detergents such as polysorbate. Although ANS dye is known to cause background fluorescence in the fluorescence spectroscopy of polysorbate containing protein formulations, it is still widely used in the tertiary structure determination as it is cost effective and the alternative dyes also has their own limitations.An extensive study, directly measuring the impact of polysorbate 80 interference on IgG1 tertiary structure determination particularly when used ANS dye is still lacking. In this study we have attempted to show that polysorbate 80 impact on the extrinsic fluorescence of a stable anti-CD20 formulation by evaluating the drug product samples before and after polysorbate removal as well as in drug substance and formulation buffer.

Materials and Methods

Extrinsic Fluorescence measurement: The IgG1 mAb drug product samples (100 µl) are diluted to 1mg/ml from the labeled concentration and 50µM ANS dye (25 µl) is added to the samples and final volume was made upto 1ml with Water for Injection (WFI). Samples (200 µl) were loaded into a 96 well black opaque plate (Costar, USA) and fluorescence spectroscopy analysis was done with excitation at 380nm and emission at the range of 410nm -600nm with an interval of 1nm. The data obtained was plotted with wavelength on X-axis and relative fluorescence units (RFU) on the Y-axis.

Polysorbate removal from the samples: The drug product test sample containing polysorbate 80 with protein concentration of 5mg/ml was prepared for analysis. Polysorbate 80 present in the drug product samples was removed using ACROSEP SDR HYPERD detergent removal columns as per the manufacturer's recommendations. Initially Columns were pre-equilibrated by passing 10ml of 150mM NaCl followed by passing 1ml of test sample through the column using a syringe. Once the sample is passed through the column, 5mL of 150mM Sodium chloride buffer was again passed and fractions are collected. A total of 8 fractions are collected with fraction 2 being ~1000 µl and the remaining fractions were ~500 µl for (fraction 1 & 3 to 8). The remaining flow through was discarded and finally 2mL of 20% ethanol was passed through the column and stored in the same solution at 2-8°C for further use.

Estimation of polysorbate in the samples after polysorbate removal step: Anti-CD20 preparation was mixed with 2.5 ml of extraction solvent in a 15 ml centrifuge tube and were mixed for 3 minutes followed by centrifugation at 4000 rpm for 2 minutes. The upper layer was collected and dried in a vacuum evaporator. A volume of 360 µl ACTC dye was added to each 2 ml centrifuge tube for derivatization with vortexing and allowed the reaction mixture cool at room temperature for 5 minutes. A volume of 360 µl dichloromethane was added to each 2 ml centrifuge tube followed by vortexing and subsequent centrifugation at 5000 rpm for 2 minutes. Finally, 200 µl of lower layer was aliquoted into a quartz plate without disturbing the upper layer and the absorbance was measured at 620 nm. Polysorbate concentration was reported as µg/ml.

All the extrinsic fluorescence data is expressed as Mean ± SD.

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Results

Extrinsic Fluorescent analysis before polysorbate removal: The IgG1 monoclonal antibody drug product (DP) was evaluated for tertiary structure analysis using extrinsic fluorescence spectroscopy. Results from this study show that the drug product samples containing polysorbate 80 along with monoclonal antibody have the emission maxima at mean RFU at 494nm and mean maximum RFU value is 24227. Whereas the same emission maxima at mean RFU (494nm) and mean maximum RFU values (21976) were observed for the DP formulation buffer (DPFB) containing polysorbate 80. The monoclonal antibody drug substance (DS) show emission maxima at mean RFU and mean maximum RFU at 512nm and 4583 respectively before formulation (See Figure 1, 2 and 3). These results clearly show the interference from polysorbate 80 in the extrinsic fluorescence based tertiary structure determination of monoclonal antibody drug product.

Extrinsic fluorescence analysis after polysorbate 80 removal: All the drug product fractions are detergent depleted and fraction 2 is considered as the major fraction based on the protein content analyzed through OD@280nm and extrinsic fluorescence. The remaining fractions 3 to 8 have less amount of protein along with the buffer. Protein recovery calculated from the 8 fractions collected is more than 85%. Polysorbate 80 estimation is done for the fractions collected and no polysorbate 80 was observed in the sample fractions analyzed for extrinsic fluorescence. The sample fraction with depleted polysorbate 80 has the emission maxima at mean RFU of 519nm and the mean maximum RFU value of 3782 for the IgG1 antibody present in the drug product (See figure 1 and 2).

Discussion

This study observed that there is difference in emission maxima and RFU intensity between drug product samples with (494nm and 24227RFU) and without polysorbate 80 (494nm and 21976RFU).

All therapeutic protein formulations including monoclonal antibody formulations contain surfactants like polysorbate 20 and 80 which are commonly used excipients to prevent protein adsorption at liquid-liquid, liquid-solid or liquid-air interfaces, which can lead to surface-induced denaturation and aggregation (6,7). Therapeutic protein based drug products especially monoclonal antibody based drugs must be thoroughly characterized for their tertiary structure during their development before release and clinical use. Extrinsic fluorescent dyes such as polarity sensitive ANS are commonly employed for the

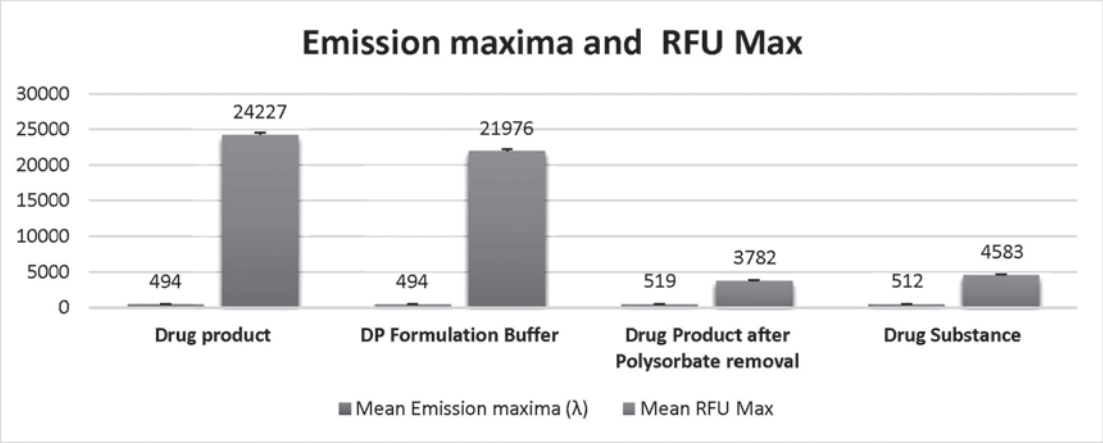

Fig. 1. Extrinsic fluorescence analysis of Drug products

Polysorbate interferes in the extrinsic fluorescence of ANS dye

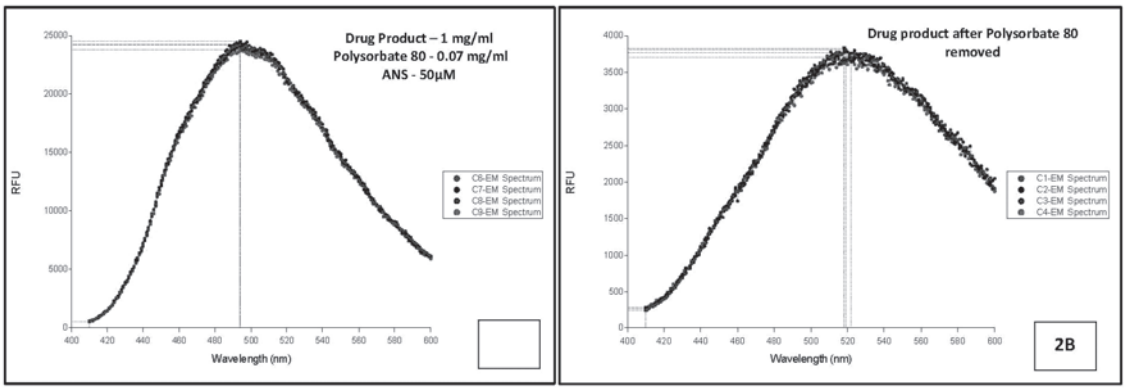

Fig. 2A & 2B. Extrinsic Fluorescence analysis of Drug product before and after Polysorbate 80 removal

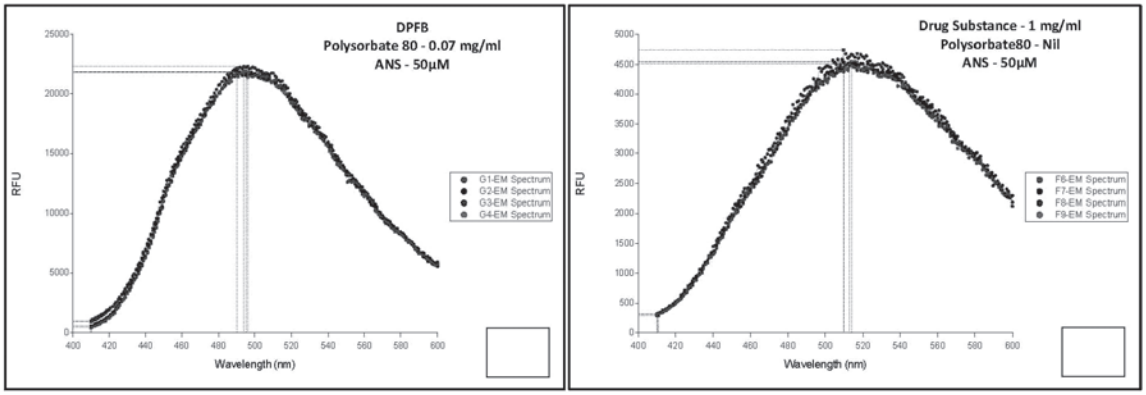

Fig. 3A & 3B. Extrinsic Fluorescence analysis of Drug substance (before polysorbate addition) and Formulation buffer (with Polysorbate)

tertiary structure determination of therapeutic proteins especially monoclonal antibodiesalthough they are known to increase background noise (8, 9). ANS dye is known to interact with polysorbate 80 and is also used for the analytical characterization of polysorbate degradation products during stability monitoring of therapeutic protein formulations (10). Avoiding or eliminating background fluorescence while using ANS dye for tertiary structure determination of therapeutic antibody drug product containing polysorbate 80 is still a challenge. In agreement to the previous reports, we also found that polysorbate 80 indeed interferes with the extrinsic fluorescence measurement of therapeutic IgG1 antibody. To know whether we can avoid polysorbate interference, in this study we removed polysorbate 80 from the drug product before extrinsic fluorescence analysis. Our results suggested that, the drug product emission maxima and RFU values are coinciding with the formulation buffer data (both containing polysorbate 80) but not with the data from drug substance and polysorbate removed drug product. This means the fluorescence observed mostly includes polysorbate 80 fluorescence in the drug product samples. This may be due to the interaction between dye-polysorbate, which results in high background fluorescence contributing to high overall fluorescence (4). New molecular rotors like

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CCVJ and DCVJ dyes are suggested as alternative to the polarity sensitive dyes while dealing with polysorbate containing formulations, but they too increase the background fluorescence when the formulations contain sugars and only suitable for high concentrated formulations (4, 11). Since, most of the monoclonal antibody formulations, especially anti-CD20 antibody formulation contain sugars as excipients, these dyes are of limited use. Alternatively, polysorbate interference can be corrected through background correction (by subtracting fluorescence of formulation buffer from drug product)or by analyzing the drug substance samples (before formulation buffer addition). But it is not an accepted practice in industryasthe tertiary structure determination should be done in formulated drug product with all the excipients to know whether the added excipients has any effect on the structure.

This study concludes that interference in extrinsic fluorescence in terms of mean emission maxima and mean RFU of IgG1 therapeutic monoclonal antibody drug product can be attributed to polysorbate 80 when ANS dye is used and polysorbate 80 removal procedure is recommended to be employed before the sample analysis.

Acknowledgements

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Conflicts of Interest : The authors declare there is no conflicts of interest.

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Formulation and Evaluation of Doxofylline Lozenges

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Abstract

Doxofylline was formulated as lozenges to provide slow release medicament for the management of asthma for cough and itchy throat. The present investigation has been taken up to design, prepare and evaluate hard candy lozenges to meet the need of improved bioavailability. The benefits of these prepared lozenges showed increase in bioavailability, reduction in gastric irritation by passing of first pass metabolism and increase in onset of action. The lozenges were prepared using sucrose as base; liquid glucose in the formulation made the lozenges transparent and smooth; hydroxypropyl methylcellulose (HPMC) and hydroxyethyl cellulose (HEC) are used as polymers. Aspartame and saccharin are used as artificial sweeteners. Sweeteners along with flavours are used to mask the bitter taste of drug. All the formulations prepared were subjected to various physicochemical parameters like hardness, content uniformity, friability, weight variation, moisture content etc. The prepared formulations have a hardness of 8-11 Kg/cm², non-gritty and pleasant mouth feel. Some selected formulations were tested for drug excipients interactions subjecting to infrared (IR) Spectral analysis. *In vitro* drug dissolution studies showed least of 82.7% for FL7 and maximum of 98.8% for FL6 release following zero order release in 30 minutes.

Keywords: Hard candy lozenges, Doxofylline, anti-asthma, polymers.

Introduction

The word “Lozenge” is derived from French word “Losenge”, which means a diamond shaped geometry having four equal sides. Development of lozenges dates back to 20th century and is still in commercial production (1). Lozenges are solid preparations that contain one or more medicaments, usually in a flavoured, sweetened base, and are intended to dissolve slowly in the mouth. In short, lozenge is a small medicated candy intended to be dissolved slowly in the mouth to lubricate and soothe irritated tissues of throat. Most of the lozenge preparations are available as over-the-counter medications. They are intended to be dissolved on the back surface of the tongue to provide drug delivery locally to the mouth, tongue, throat, etc. to minimize systemic and maximize local drug activity. The dosage form can be adopted for local as well as systemic therapy and a wide range of activities can be incorporated in them. They can deliver drug multi- directionally into the oral cavity or to the mucosal surface (2). Lozenges currently available in market are of four types: caramel based medicated lozenges, soft lozenges, hard candy lozenges and compressed tablet lozenges. Hard candy lozenges are prepared by moulding. Moulded lozenges are sometimes referred to as pastilles, whereas compressed lozenges prepared on tablet compression machine, may be referred to as troches (3).

Lozenges are placed in oral cavity. Since the sublingual lozenges may be impractical due to their size, buccal lozenges are formulated and have been extensively used and are intended to be placed between the cheek and the gums. Though the lozenge dissolution time is about 30 minutes, it also depends on the patient, as patient

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controls the rate of dissolution and absorption by sucking on lozenge until it dissolves. Sucking and the subsequent production of saliva may also lead to increased dilution of the drug and accidental swallowing (4).

Drug candidates which can be incorporated in lozenges belong to one of the following categories: antimicrobials and local anaesthetics for throat pain; aromatics, herbals, zinc salts, decongestants, anti-histamines and cough suppressants for cold, allergy, cough, congestion and nicotine like substances for smoking cessation.

Materials and Methods

Doxofylline was a gift sample from Hetero Labs, Hyderabad. Hydroxypropyl methylcellulose (HPMC) K4M and hydroxyethyl cellulose (HEC) were purchased from the S D Fine-Chem Limited, Mumbai. Aspartame was a gift sample from Merck Ltd., Mumbai, India. Sucrose, liquid glucose, colour and flavour from local chemical suppliers.

Method of Preparation : Hard candy lozenges are prepared by heating and congealing technique. Sucrose is accurately weighed and is dissolved in one third amount of water by heating on fire cookers until all sugar granules are dissolved. Liquid glucose is added when cooking temperature reaches 110^{o}C and heating is continued until final temperature is 145^{o}C to 156^{o}C. The mixture is cooled to 135^{o}C and colour is added. Further, cooling is carried out and mixed until temperature reaches 40^{o}C. The flavour, drug and polymer are added and mixed for 4 to 6 minutes and poured in lubricated moulds (5). These prepared lozenges are further subjected to various evaluation parameters. The formulations of prepared lozenges are shown in table 1.

Evaluation of Lozenges

Physical parameters : The general appearance of a lozenge, its visual identity and overall elegance is essential for consumer acceptance, control of lot-to-lot uniformity, tablet-to-tablet uniformity and monitoring trouble-free manufacturing. It involves the measurement of attributes such as size, shape, colour, surface texture and consistency.

Thickness Test : The thickness in millimeters (mm) was measured individually for 10 pre-weighed lozenges by using a Vernier Calipers. The average thickness and standard deviation were reported. The thickness of a lozenge can vary without any change in its weight. This is generally due to the difference of density of granules, pressure applied for compression and the speed of compression. The thickness variation limits allowed are 5% of the size of the tablet.

Diameter : The diameter size and shape of candies depend on the moulds. The lozenges of various sizes and shapes are prepared but generally they are circular with either flat or biconvex faces (6).

Weight Variation Test : Twenty (20) lozenge from each batch were individually weighed in grams on an analytical balance. The average weight and standard deviations were calculated. Individual weight of each lozenge was also calculated using the same and compared with average weight. If any weight variation is there, that should fall within the prescribed limits (generally 10% for lozenge weighing 120 mg or less, 7.5% for lozenge weighing 120 mg to 300 mg and 5% for lozenge weighing more than 300 mg):

% Deviation= (Individual weight - Average weight / Average weight) × 100

Hardness Test : The hardness of lozenges was measured using a Monsanto Hardness Tester. The crushing strength of the 10 lozenge with known weight and thickness of each batch was recorded in kg/cm^2 and the average hardness and the standard deviation was reported. The hardness of lozenge depends on the weight of the material used, space between the upper and lower punches at the time of compression and pressure applied during compression. The hardness also depends on the nature and quantity of excipients used during formulation (7).

Friability Test : Twenty (20) lozenges were selected from each batch and weighed. Each group was rotated at 25 rpm (rotations per minute) for 4 minutes (i.e.100 rotations) in the Roche friabilator. During each revolution, the lozenge fall from a distance of six inches to undergo a shock. The

Table 1: Formulae to prepare hard candy lozenges

INGREDIENTS (mg)	FORMULATION CODE									
	FL1	**FL2**	**FL3**	**FL4**	**FL5**	**FL6**	**FL7**	**FL8**	**FL9**	**FL10**
Drug (mg)	200	200	200	200	200	200	200	200	200	200
HPMC K4M	12	24	30	60	-	-	-	-	-	-
HEC	-	-	-	-	12	24	30	60	-	-
Saccharin sodium	90	100	100	95	74	90	95	90	114	95
Aspartame	90	116	140	109	100	120	130	110	120	120
Sucrose	1893	1851	1824	1845	1899	1857	1859	1849	1845	1857
Liquid glucose	854	848	845	830	854	848	845	830	860	840
Preservative	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Colour	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Flavour	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

lozenge were then dusted and re-weighed to determine the loss in weight. Friability was then calculated as per weight loss from the original tablets. Compressed lozenge that lose less than 0.5 to 1 % of weight are generally considered acceptable (8):

% Friability = Initial weight – Final weight / Initial weight x 100

Drug Content Uniformity : The content uniformity was tested by powdering one lozenge and dissolving the powder content in 100 ml volumetric flask containing 50 ml of 6.8 phosphate buffer and allowed to stand for 30 min. The mixture was made up to volume with buffer pH 6.8. The diluted samples absorption was recorded at 274 nm. For most of the larger dose drugs in lozenge form, the official potency range permitted is not less than 95% and not more than 105% of the labelled amount (9).

Moisture Content : By Gravimetric method, one gram sample is weighed and placed in vacuum oven at 60-70°C for 12-16 hrs. Final weight is subtracted from initial and the difference in moisture content is calculated:

% Moisture content = Initial – Final weight × 100

***In vitro* Drug Release Studies :** The rate of the drug absorption was determined by the rate of drug dissolution from the lozenges. Thus, the rate of dissolution and bioavailability may be directly related to the efficacy of the tablet lozenge. The *in vitro* drug release study was performed for the prepared lozenges using USP Apparatus II (Paddle type). A 250 ml of the dissolution medium phosphate buffer pH 6.8 was placed in the beaker containing the lozenge and stirred at 100 rpm. A 5 ml aliquot sample was withdrawn at 5 min and replenished with same volume of fresh media. The drug content in the samples was estimated using UV-spectrophotometer at 274 nm (10).

Fourier Transform Infrared (FTIR) Spectroscopy : The Fourier Transform Infrared (FTIR) spectra of samples were obtained using FTIR Spectrophotometer (Perkin Elmer) for drug excipients compatibility. Pure drug, individual polymers and optimised formulations were subjected to FTIR study. About 2–3 mg of sample was mixed with dried potassium bromide (KBr) of equal weight and compressed to form a KBr disk. The samples were scanned from 400 to 4000 cm^{-1} (12).

Standard graph of Doxofylline at 274 nm : Standard stock solution of pure drug containing 100 mg of Doxofylline/100 ml was prepared using different buffer solutions like 6.8 pH phosphate buffer. The working standards were obtained by dilution of the stock solution. The standard curves for Doxofylline were prepared in concentration range of 2-12 µg/ml at the selected wave length

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274 nm. Their absorptivity values were used to determine the linearity. Solution was scanned and Beer-Lambert law limit was obeyed in the concentration range of 2, 4, 6, 8, 10 and 12 µg/ml (figure 1).

Results

Construction of Calibration curve : The study started with the construction of standard calibration curve. The λ_{max} of Doxofylline in 6.8 pH phosphate buffer was scanned and found to have the maximum absorbance at 274 nm. The standard graph of Doxofylline in 6.8 pH phosphate buffer was plotted by taking concentration ranging from 2-12 µg/ml and a good correlation was obtained with R^2 values of 0.996 respectively.

Drug Excipient Compatibility Studies : The compatibility between the drug, polymer and excipients was compared by FTIR spectroscopy (Perkin Elmer). FTIR spectrum of pure drug exhibits characteristic peaks at 3108.20, 1692.45, 1427.09 and 1010.35 cm^{-1} due to O-H, C-O, C-H and N-H stretching respectively. FTIR spectrum of optimized candy mixture showed characteristic peaks at 3310.35, 1657.62, 1426.96 and 984.80 cm^{-1}. The presence of above peaks confirm undisturbed drug in the formulations. It was observed that, there was no disappearance or shift in peak position of drug in any spectra of drug and polymers which proved that drug and polymers were compatible. Hence, it can be concluded that drug can be used with the selected polymers and excipients without causing instability in the formulation. FTIR data interpretation of drug and formulation was shown in table 4.

Evaluation parameters of Hard Candy Lozenges : All the prepared formulations were tested for physical parameters like weight variation, hardness, thickness and diameter are found to be within the Pharmacopoeia limits. The results of the tests were tabulated and was shown in table 2.

The total weight of each formulation was maintained constant; the weight variation of the lozenges was within the permissible limits of 5%. Weight of the tablet was fixed at 3.00 grams and the weight variation for every batch was tested. The formulations FL1, FL2, FL3 and FL4 containing HPMC were weighed and the increase in weight variation order is FL3< FL4 <FL2<FL1. For the formulation containing HEC the increasing order of weight variation is FL8<FL7<FL6<FL5. The formulation containing no polymer was also weighed and all the formulations were found within the acceptance limits.

Hardness of the tablet was maintained at 10.2-11.5 kg/cm^2 for all the batches. The highest hardness was found in formulation FL4 i.e.11.51±0.29 kg/cm^2 and least hardness was determined in FL2 i.e.10.2±0.64 kg/cm^2. Since there is no specified standard limits for the deviation in the hardness of lozenges, comparing each formulation with one

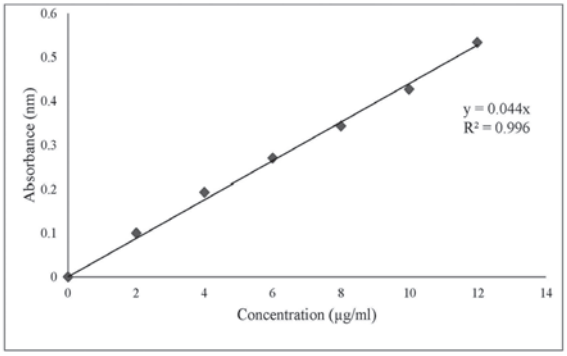


Fig. 1: Standard graph of Doxofylline in phosphate buffer pH 6.8

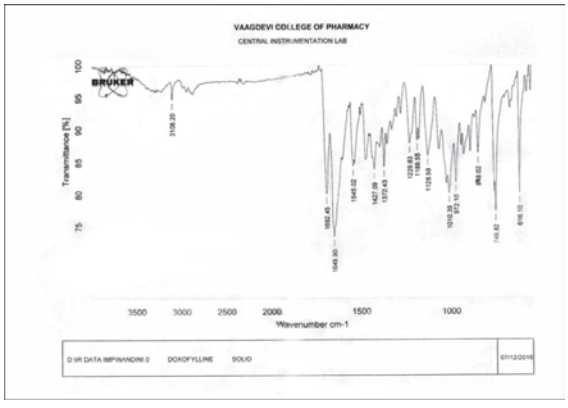


Fig. 2: FTIR Spectrum of Doxofylline

another it could be concluded that, because the difference between the standard deviations are not too large, the formulations had good uniformity in the hardness.

The thickness and diameter values were almost uniform in all the ten formulations. It was found to vary between 7.25 to 7.43 mm and 19.12 to 19.19 mm. Friability was not conducted for candies as they are sufficiently hard to resist the mechanical abrasion.

Moisture Content : The moisture content of all the lozenges was within 2%. The results are shown in table 3.

Drug Content : The drug content was calculated for all the prepared formulations. Table 3 shows the result of drug content of each formulation. Three replications of each test were analyzed for mean and standard deviation. The drug content found in the formulations containing HPMC was found to be highest for FL1 and lowest for FL2 and for HEC containing formulations the drug content is highest

Table 2 : Evaluation of Doxofylline Lozenges prepared with varying concentration of different polymers

FORMULATION CODE	WEIGHT VARIATION (mg)	HARDNESS (kg/cm^2)	THICKNESS (mm)	DIAMETER (mm)
FL1	3000.9±3.1	10.41±0.41	7.25±0.01	19.17
FL2	3000.7±1.3	10.54±0.45	7.38±0.04	19.16
FL3	2999.2±2.3	10.79±0.52	7.29±0.03	19.17
FL4	2999.7±2.8	11.51±0.29	7.36±0.01	19.17
FL5	3000.5±2.0	10.9±0.42	7.43±0.04	19.19
FL6	2999.8±3.5	10.2±0.64	7.33±0.05	19.12
FL7	2999.5±1.4	10.8±0.9	7.29±0.04	19.17
FL8	2999.3±2.4	10.4±0.41	7.39±0.03	19.19
FL9	3000.6±1.4	11.3±0.72	7.38±0.01	19.17
FL10	2999.8±2.3	10.7±0.51	7.42±0.04	19.14

Table 3: Moisture content and drug content of Doxofylline Lozenges

FORMULATION CODE	MOISTURE CONTENT	DRUG CONTENT (%)
FL1	0.92±0.05	99.5±1.64
FL2	0.84±0.07	98.34±1.47
FL3	0.83±0.05	98.7±2.0
FL4	0.87±0.07	98.8±1.57
FL5	0.86±0.08	99.6±1.41
FL6	0.87±0.07	99.8±1.45
FL7	0.87±0.05	98.5±1.71
FL8	0.85±0.07	99.3±1.64
FL9	0.85±0.05	99.6±1.94
FL10	0.85±0.05	98.33±1.46

for FL6 and lowest for FL7.The formulation without polymer i.e.FL9 and FL10 contain 99.6±1.94% and 98.33±1.46% of drug and hence all the formulations were found to be within the standard limits.

***In vitro* release data for candy based Lozenges :** All the ten formulations prepared were subjected to *in vitro* release study. The *in vitro* method for studying the release rate is maintained so that it must simulate the mouth condition. In the present work *in vitro* release study was carried out using dissolution apparatus. For different time interval, sample was withdrawn and cumulative drug release was calculated. The dissolution apparatus USP II (Paddle type) was used. The temperature was maintained at 37±0.5°C and stirred at 50 rpm. The dissolution medium is 6.8 pH phosphate buffer. The samples were withdrawn at 5 min interval for 30 min.

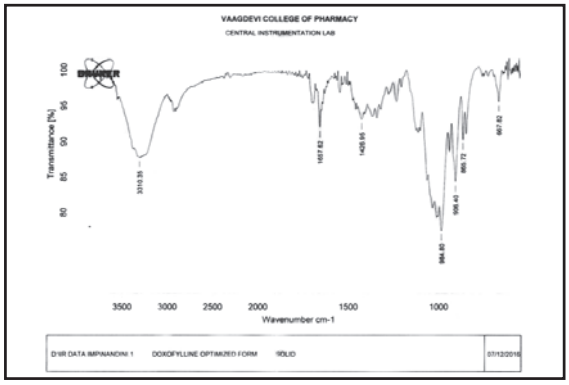


Fig. 3: FTIR Spectrum of Optimized formulation

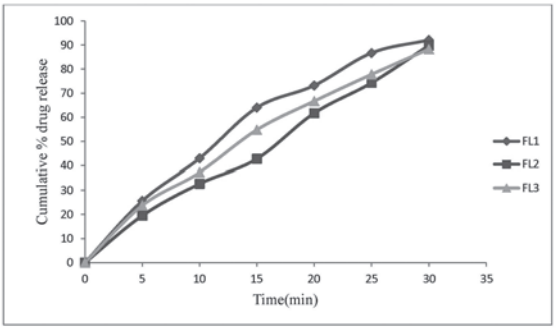


Fig.4: Graphical representation of cumulative percent of Doxofylline release from Lozenges

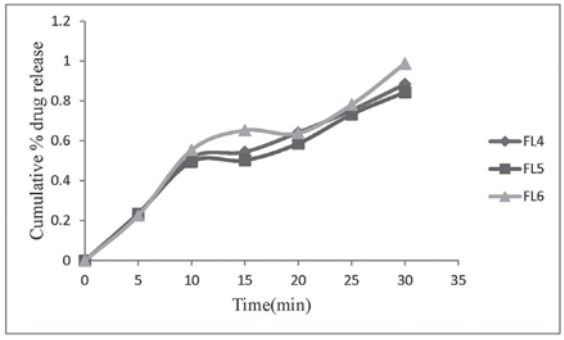


Fig. 5: Graphical representation of cumulative percent of Doxofylline release from Lozenge

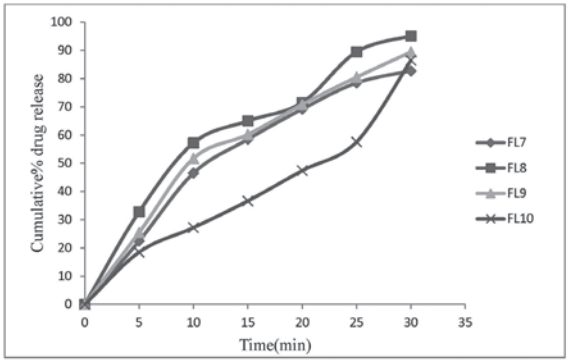


Fig. 6: Graphical representation of cumulative percent of Doxofylline release from Lozenges

Cumulative percentage drug release was calculated on the basis of mean amount of Doxofylline present in the respective lozenges. The results are given in table 5, 6, 7 and figure 4, 5, and 6. The cumulative percentage drug release of FL1 and FL8 was 86.7% and 89.5% respectively within 25 min and for FL7, it was 82.7% at the end of 30 min. For FL6, the drug release was 98.8% at 30 min. The cumulative percentage drug release of FL2, FL3, FL4, FL5, FL9 and FL10 was 89.8%, 88.3%, 88.5%, 84.4%, 89.44% and 86.5% respectively by the end of 30 min. Hence by the determination of the *in vitro* release data, it can be concluded that, the drug release was faster in case of FL1, FL6 and FL8.

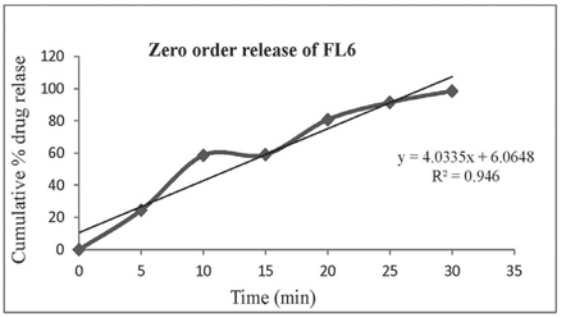
Drug Release Kinetics of Doxofylline : The release kinetics and correlation coefficients were calculated for all the optimized formulations. All

Table 4: FTIR studies were carried out for pure drug along with drug and excipient combination

FTIR SPECTRA	O-H STRETCHING	C-O STRETCHING	C-H STRETCHING	N-H STRETCHING
Drug	3310	1693	1427	1010
Optimized formulation	3310	1692	1426	984

Table 5: Cumulative percent of Doxofylline release from lozenges containing polymers

Time (min)	FL1 (Mean) SD	FL2 (Mean) SD	FL3 (Mean) SD
0	0	0	0
5	25.6%	19.5%	23.7%
10	43%	32.7%	37.3%
15	64%	42.8%	54.8%
20	73.2%	61.7%	66.7%
25	86.7%	74.3%	77.8%
30	92.1%	89.8%	88.3%



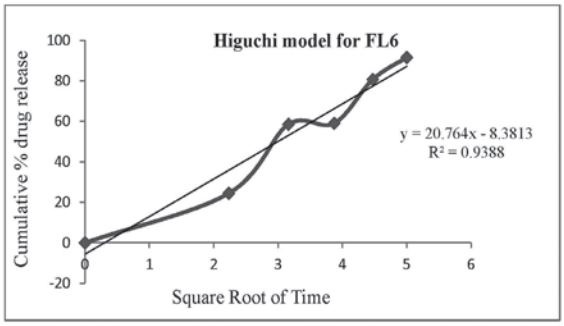


Fig. 7: Release plots for FL6.

Table 6: Cumulative percent of Doxofylline release from lozenges containing polymers

Time (min)	FL6 (Mean) SD
0	0
5	22.6%
10	55.4%
15	65.3%
20	63.8%
25	78.2%
30	98.8%

the formulations followed Higuchi profiles with R^2 values more than 0.9, followed by zero order which account for the diffusion controlled release.

Discussion

The objective of this study was to formulate and evaluate Doxofylline lozenges for anti-asthmatic activity suitable for patients suffering from cough and itchy throat.

The drug content of all formulations was between 98.3 to 99.8% indicating the presence

Table 7: Cumulative percent of Doxofylline release from lozenges containing polymers

Time (min)	FL7 (Mean) SD	FL8 (Mean) SD	FL9 (Mean) SD	FL10 (Mean) SD
0	0	0	0	0
5	22.45%	32.8%	25.45%	18.5%
10	46.5%	57.2%	51.72%	27.2%
15	58.5%	65.01%	60.2%	36.65%
20	69.2%	71.5%	70.85%	47.39%
25	78.5%	89.5%	80.49%	57.57%
30	82.7%	95.1%	89.44%	86.5%

of an acceptable amount of drug in the formulations. Furthermore, all the formulations showed acceptable weight variation, hardness, thickness and moisture content. The aim of the study was to show good oral retention time and moderate release of the drug for a period of 20 to 30 min. All the formulations containing the polymers showed good oral retention time of 20-30 min. Change in the physical form of the lozenge was observed within 10-15 min for candies. The drug release rate study revealed that release from HEC containing formulations was found to be rapid and maximum for candies compared to HPMC K4M. Increase in the concentration of polymers increased the drug release but further increase has led to decrease in the drug release. Initial low release rate might be due to fast rate of hydration followed by quick gelation and further release rates might be due to diffusion through the hydrated polymer layers.

For any formulation, drug excipient interactions play an important role and hence the formulations were subjected to infrared spectral analysis, it was observed that undisturbed drug peaks revealing the compatibility drug.

The suggested ratio of the sugar to liquid glucose is 60-40% for attaining transparency and smoothness. This is due to prevention of sugar crystallization by liquid glucose.

But in the present investigation, sufficient transparency was attained with the use of sugar to liquid glucose 13%, 18% and 20%. This suggests that even low concentration of liquid glucose has the ability to retain the capacity to prevent crystallization of sugar.

This difference in the concentration of liquid glucose to attain the smoothness and transparency may be due to the type of apparatus used in the cooking process: 20% open kettle, 30% batch vacuum cookers, 35% semi-continuous, and 40% continuous cookers.

The difference in the requirements of liquid glucose is due to increasing amount of mechanical action or turbulence to which the candy is subjected after cooking like more agitation and more requirement of liquid glucose.

Other mechanisms to control the crystallization are:

a. High molecular weight sugar in the liquid glucose

b. Low cooking temperatures

c. Minimum mixing during cooking.

Dextrose is used instead of liquid glucose (12). Use of 40% dextrose instead of liquid glucose effected the transparency. This may be due to failure of dextrose to retard crystallization of the sugar. Even use of gelatin, which was transparent when heated with water (forms transparent soft gel like consistency) also failed to attain the transparency alone as well as combination with liquid glucose. Use of honey instead of liquid glucose resulted in the transparent lozenges but was not satisfactory. The formulation developed

using honey was sticky due to hygroscopic nature of honey. The obtained transparency with honey is due to its ability to retard crystallization.

Conclusion

Patient compliance is one of the important aspects for administration of drug especially those which are bitter in taste. In the present study, Doxofylline sweetened hard candy lozenges were designed for the effective treatment of asthma.

The interest was for the development of new dosage form and the effect of different concentration on the *in-vitro* release. The estimation of drug by UV spectrophotometer was carried out. The possible interaction between the drug and excipient was studied by FTIR spectroscopy, which showed that there was no interaction between the selected drug and polymer under study.

Lozenges could be successfully prepared by heating and congealing method using sucrose, liquid glucose, aspartame, polymers, dextrose, flavour and colour.

In vitro release rate studies showed that the drug release for lozenges was maximum in formulation FL6 (98.8±1.57%) which was at 30 min.

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Plant growth promotion by bacterial inoculum with different conditions and estimation of enzyme activity from germinating seeds and bacterial isolates

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Abstract

Plant health and other parameters are dependent on plant growth promoting (PGP) abilities which are further dependent on the micro-flora in the plant rhizospheric soil. In this study both the sterile and non-sterile soils of Dehradun were taken for the PGP and the seeds of *Cicer arietinum* were taken for the vigour index with the treatment of NKA1 (phosphate solubilizer). Our results showed that the VI of NssT[†] (1599.532) &NssT[*] (1630.729) were high as compared with SsT[†] (1069.866) &SsT[*] (606.554). When the dry weight of roots and shoots of *Cicer arietinum*were calculated there were no significant difference in these treatments. Other study was focused on estimation of â-amylase activity of germinating seeds and á-amylase activity of 26 bacterial isolates. NKA1 and isolates genomic DNA was isolated by the phenol-chloroform DNA extraction method and DNA has no traces of contaminants. Overall study showed that plants are favorable for the growing in natural soil with potential microbes. Rhizobacterial population mainly promote & helps the plant growth via different mechanism and microbial inoculants improve plant health and yield.

Keywords: PGP, *Cicer arietinum* L., vigor index, enzyme activity.

Introduction

Microbial inoculants are called plant growth promoting (PGP) regulators contain bacterial isolates from genera such as *Pseudomonas, Enterobacter, Rhizobium, Azospirillium, Azotobacter* and *Bacillus*(4, 7, 19) well recognized and a great important component of integrated plant nutrient and health management for sustainable agriculture and also hold great promise to improve crop growth and yield (11). Plant growth promoting rhizobacteria (PGPR) accounts for about 2-5% of total the rhizobacteria involved in plant growth promotion (2). Rhizospheric bacteria mainly promote plant health, (5) they stimulate plant growth directly and indirectly by produce or change the concentration of plant growth regulators (10, 16). Present research suggested that several *Pseudomonas sp.* and *Bacillus sp.* have been reported among of the most efficient phosphate solubilizing bacteria and as it important bio inoculants due to their multiple bio fertilizing activities of better improvement soil nutrient status with secretion of plant growth regulators and suppression of soil borne pathogens (21, 22). Non-rhizosphere or unfertile soil can be unfavorable for microbial growth, and introduction of bacterial suspensions often results in rapid decreases in population size and activity in plant growth (20). Studies on plant growth in autoclaved soils generally pertain to effects of microbial inoculations, whereas only few deal with intrinsic effects of soil sterilization on plant growth (13). Recent research suggested that enzymes and hormones have play critical role in removing germination and dormancy of seeds and amylases enzyme in aleurone layers is believed to be essential for seed germination in seeds, which is tightly regulated by hormones

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synthesized in the embryonic stage (8). Two major classes of amylases (á-amylase & â-amylase), have been identified in microorganisms and germination seeds. Amylase enzyme produced by several fungi, yeasts, bacteria, seeds and actinomycetes. However, enzymes from different sources have dominated applications in industrial sectors and research and *Cicer arietinum* L. was selected for pot trials to check the efficacy of plant growth promotion by screened PGPR under different treatment and soil condition.

Materials and Methods

The soil used in experiments was collected from an experimental field near Graphic Era University, Dehradun (Latitude: 30°-30°32', Longitude: 77°43'-78°24'). The soil was autoclaved at 121°C / 15lb for 30min (3times). Sterile soil sample was plated on nutrient agar by serial dilution (double distilled water)for the checking of proper sterilization of soil. *Cicer arietinum* L. (Chickpea), *Hordeumvulgare*L. (Barley) and *Phaseolus vulgaris* (Rajma) seeds used for the enzyme activity experiments were obtained from Dehradun, Uttarakhand, India (Fig.3). For the other study, rhizospheric soil samples of *Hibiscus rosa-sinensis*, *Menthaspicata*, *Ficusreligiosa* and *Oryza sativa* were collected for the isolation of PGPR.

Microbial inoculum was isolated from the rhizospheric soil sample of *Hibiscus rosa-sinensis*. This bacterial isolate (NKA1) was selected for the ability of indol-3-acetic acid (IAA) production and phosphate solubilizer (PS). Firstly, ten rhizobacteria were isolated from the soil. Then, highly IAA and PS performing isolate was selected for the treatment of sterilization and non-sterilization effect on plant growth. NKA1 used as a bio-inoculum for high ability in phosphate solubilization and indole production, based on that NKA1 was treated in the process named seed treatment and soil treatment to see the effects on plant growth promotion in sterile and non-sterile soil treatment condition. Fig. 1 suggested that, depending on thetreatment condition and inoculum preparationplant growth can be enhanced.

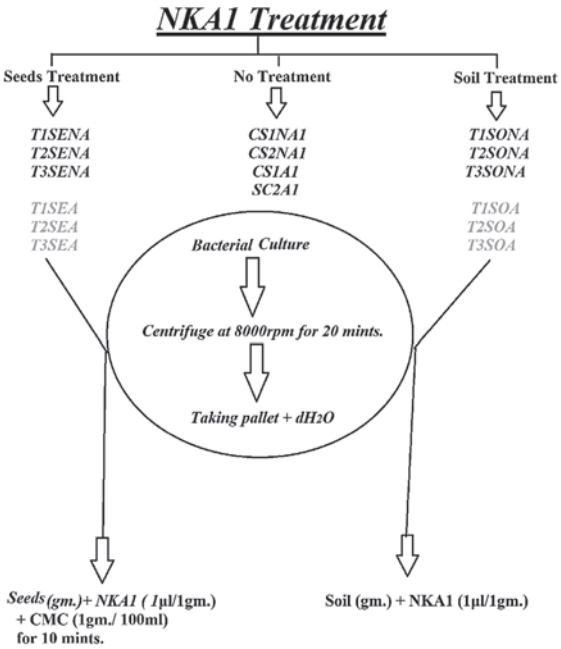

Fig. 1. Inoculum preparation and treatments

NKA1 was inoculated into sterilized Peptone broth containing 1% of tryptone for 48hrs. at 28^{0}C. After 48 hrs., 1 ml of kovac's reagent (HiMedia, India) was addedto all the tubes including controland shaken gently (6).

Phosphate solubilization ability of the isolate was evaluated in Pikovskaya's (PKV) agar medium (HiMedia, India) incorporated with tri calcium phosphate (TCP) as insoluble phosphate. Spot inoculation was done in PKV plate for 24 hrs.at 28^{0}C. Positive phosphate solubilizing bacteria showed the clear zone around the colony (9).

Plant growth promotion (PGP) by NKA1 apply on *Cicer arietinum* L. as following seeds with different soil treatments in sterile and Non-sterile condition. The seeds were surface sterilized in 70% ethanol for 15 seconds, 2% sodium hypochloride for 3 minutes followed by 5 times washing in sterile double distilled water.

The TZ (Tetrazolium test) or viability test is a biochemical test, which differentiates live from dead seeds based on the activity of the respiration

enzymes in seeds. 30 seeds were placed on 30ml TZ solution (1% 2, 3, 5 triphenyltetrazolium chloride, Sigma-Aldrich, India) in dark room and 10 seeds were taken as control without TZ solution for 48 hrs.forthe germination assay (15).

The experiment was study on the basic of plant growth in sterile and non-sterile soil. In this experiment, CS1NA1, CS2NA1, CS1A1 and SC2A1 are the control set of each experiment and every set maintained triplicate with plant. T1SENA, T2SENA & T3SENA were maintained by seeds + NKA1 (1µl/ 1ml) + CMC (1gm/100ml) for 10 minutes in non-sterile soil condition and T1SEA, T2SEA & T3SEA followed same treatment condition in sterile soil. On the present of growing of *Cicer arietinum* L., in soil (gm.) + NKA1 (1µl/ 1ml) treatment condition, naming of the plants are T1SONA, T2SONA & T3SONA in non-sterile soil and T1SOA, T2SOA & T3SOA in sterile soil. (Treatment flow chart: Fig. 2).

Seedling vigor index was calculated as per the recommendations of ISTA (1976). Seeds were placed in between two wet filter paper, 30 seeds each were placed equidistantly and kept for incubation at 25±2^{0}C for 48 hrs.. After 2 days of incubation, seeds were evaluated for germination percentage. Observations were recorded for the seed germination. Shoot length, root length and germination percentage were calculated. The length and shoot length was measured in centimeter scale and vigour index was calculated by adding mean root length and shoot length and multiplied by percentage of germination (1).

The seeds were surface sterilized in 2% sodium hypochlorite for 5 min and followed 10 times washing in sterile double distilled water before the start of germination test. Seeds were placed in 90 mm diameter disposable petri dishes on layers of absorbance cotton that was

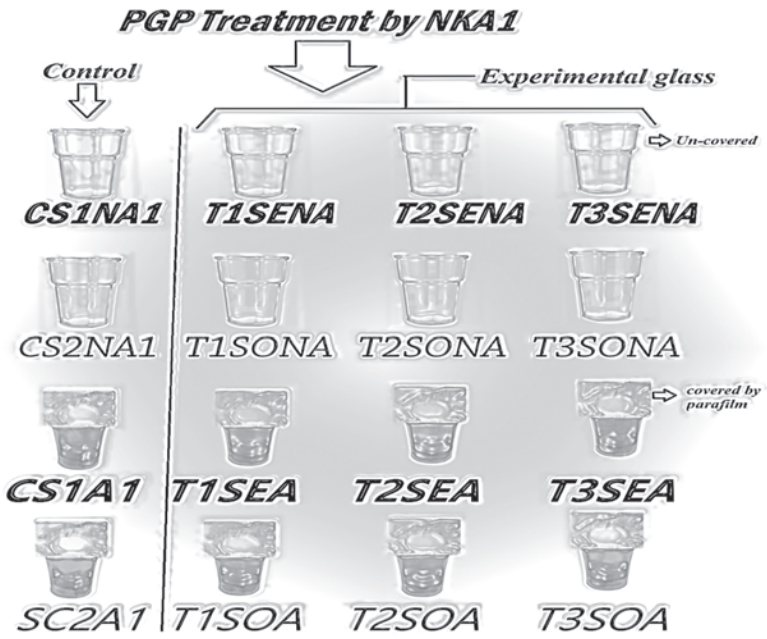

Fig. 2. PGP treatment by NKA1 in sterile and non-sterile condition (CS1NA1, CS2NA1, CS1A1 & SC2A1: Control of sterile & non-sterile set; T1SENA, T2SENA & T3SENA: Set of seeds treatment exp. in non-sterile soil; T1SONA, T2SONA & T3SONA: Set of soil treatment exp. in non-sterile soil; T1SEA, T2SEA & T3SEA: Set of seeds treatment exp. in sterile soil; T1SOA, T2SOA & T3SOA: Set of soil treatment exp. in sterile soil.

Table. 1. a. *Cicer arietinum* L. (Chickpea)

Set : 1		**Set : 2**	
Single seed weight	0.511gm	Single seed weight	0.350gm
Total seeds weight	12.683gm	Total seeds weight	12.186gm
Total germination seeds	30	Total germination seeds	30

Table. 1. b. *Hordeumvulgare*L. (Barley)

Set : 1	
Germinating seed	1.408gm
Non-germinating seeds	2.390gm
Total seeds	159^{a} + 180^{b}

a: germination seeds, b: non-germination seeds

Table. 1. c. *Phaseolus vulgaris* (Rajma)

Set : 1		**Set : 2**	
Single seed weight	0.825gm	Single seed weight	1.117gm
Total seeds weight	39.844gm	Total seeds weight	22.101gm
Total germination seeds	30	Total germination seeds	30

moistened with 7 ml distilled water. Then, petri dishes were put in a dark room with germination temperature (30°C, 24hrs.). Infected seeds were removed during counting. After that time note down the data of seeds germination and weight (8) (Table. 1.a, 1.b, 1.c).

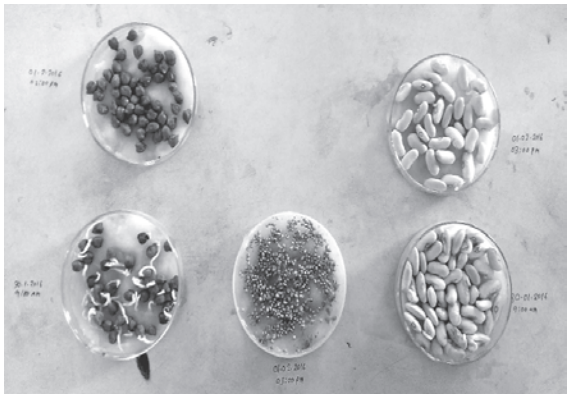

Fig. 3. Experimental seeds

Bacterial isolates á-amylase activity was assayed by 1.0 ml enzyme with 1.0 ml starch solution (1.0%) prepared in 0.1 M phosphate buffer, pH 7.5. After incubation (50°C for 5 min.), the reaction was stopped and the reducing sugars released were determined by the addition of 1.0 ml of 3,5-dinitrosalicylic acid reagent with maltose as standard (14,17). The final volume was made to 5 ml with distilled water and the absorbency measured at 540 nm with a spectrophotometer (Thermo Scientific™ GENESYS 10S UV). Macerate *Cicer arietinum* (12.683gm + 12.186gm = 24.869gm), *Hordeumvulgare*(1.408gm + 2.390gm = 3.798gm) and *Phaseolus vulgaris* (39.844gm + 22.101gm = 61.945gm) of washed seeds in a chilled pestel and mortar with 5ml of 0.05M phosphate buffer (pH 7.0) containing 0.5M NaCl. After properly crashing the seeds were centrifuged at 10,000×g for 15 minutes at 4^{0}C in cooling centrifuge (Eppendorf, 5810R). Decant the supernatant and used it as the enzyme

preparation. Then, pipetted 1ml of starch solution and 1ml of an appropriately diluted enzyme preparation in two tubes. In one of the tubes, which served as a zero min control, terminated the reaction immediately by adding 2ml of Di-Nitro-Salicylic Acid (DNSA) reagent. Incubated the other test tube at 37^0C for 15 minutes and then stop the reaction by adding 2ml DNSA reagent. After that time, kept the tubes in a boiling water bath for 5 minutes and then added 1ml of sodium potassium tartarate to each of the tubes. Cooled the tubes under running water, make up the volume up to 10ml with double distilled water and readings were taken at 540nm using UV-Visible spectrophotometer (Thermo Scientific™ GENESYS 10S UV). Standard graph was finally prepared by plotting concentration of maltose in mg/ml Vs Optical Density at 540nm (18). The protein concentration of the germination seeds was determined by the Lowry method with bovine serum albumin (BSA) as standard(12).

Genomic DNA was isolated from NKA1 and 26 bacterial isolates according to the following procedure. 10mL nutrient broth was inoculated with a single bacterial colony of NKA1 for 48hrs. at 37^0C. Cells were collected by centrifugation at 10,000 rpm, 4°C, for 10 min. Resuspend the pellet in 450µl of TE buffer (10mM Tris-HCL, pH 8.0 and 1mM EDTA, pH 8.0) and added 45µl of 10% SDS + 10µl of proteinase K and incubated for 1hrs. at 37^0C. After the incubation, added 500µl of Phenol-chloroform (1:1), mixed properly and centrifuged the mixture at 10,000rpm for 3minutes. The upper aqueous phase was transferred to a new tube, added 500µl Phenol-chloroform (1:1) and again centrifuged the mixture at 10,000rpm for 5minutes. The upper aqueous phase was transferred to a new tube and added 50µl of sodium acetate + 300µl of isopropanol to precipitate the DNA. Spool out the DNA and added 1mL of 70% ethanol for 30 second and centrifuged briefly. Drain the tube on a paper towel and re suspend the DNA in 150µl TE buffer at 4^0C. To check the genomic DNA was properly done. DNA sample was run in 0.8% agarose gel electrophoresis (1X TAE) at 80 volts for about 40 minutes and it was observed in a gel documentation system.

Results and Discussion

The effects of soil sterilization process was checked by the nutrient agar platting method after the soil autoclaved. Sterile and non-sterile soil sample were taken for the serial dilution (10^{-5}) and diluted sample was plated onto nutrient agar media (NAM). After 48hrs., observed the plate and confirmed that bacterial colony appeared in the non-sterile soil and no growth in sterile sample (fig.4).

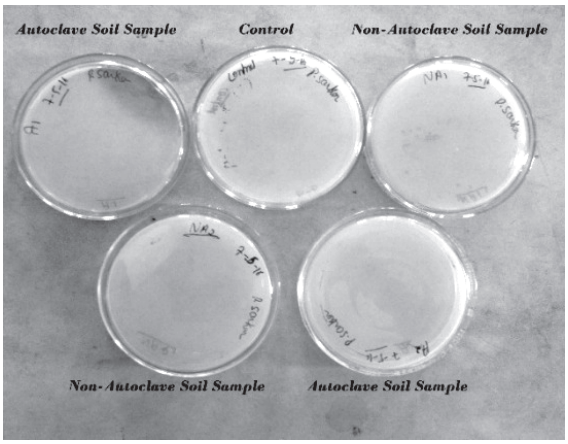

Fig. 4. No microbial growth on plate after sterilization of soil

NKA1 isolated bacterial strain is a highly potential and effective activity in IAA production and phosphate solubilization. Phosphate solubilizing ability of the isolate was checked on Pikovskaya's (PVK) agar medium incorporated with $Ca_3(PO_4)_2$. Formation of halo zone around 20.5mm the developing colonies indicated phosphate solubilizing ability of the isolate. After incubation, adding the Kovac's reagent a pink colour band was appeared at the junction of medium and reagent. The appearance of pink colour band indicates that IAA production positive by the isolated strain.

Following the ISTA (1976) recommendations, test seeds germination results showed that the germination of first plate is 73.33% and another second plate is 86.66%. Mean value of the *Cicer*

arietinum L. (experimental seeds) germination is 79.99% (5.A &5.B). In another experiment, Seeds are placed in a TZ solution for 2 days. During this process hydrogen ions reduce the colorless TZ solution to red formazan, which stains live tissues with red color while dead tissues remain unstained (3). In our study, all seeds are transformed into red colored that indicates viable seeds and it's showed perfect results in experiment (5.C).

Soil sterilization is frequently used to eliminate and reduce the microbial activity. In this studies showed that plant growth in sterile soil and non-sterile soil by NKA1 was showed highly affective results. Growth rate is low in sterile or autoclaved soil, on the other side in non-sterile or non-autoclaved soil growing plant has able to increase the vigor index better then $SsT^{\dagger}$&SsT^{*}(Fig.6). This experiment showed that

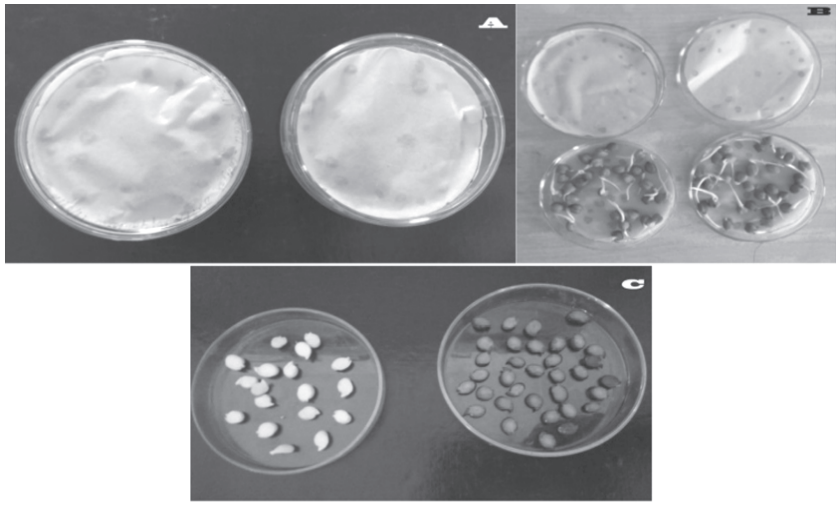

Fig. 5. Seed germination and Viability test; A & B: seed germination of *Cicer arietinum* L.; C: Experimental seeds viability determination by Tetrazolium test (TZ)

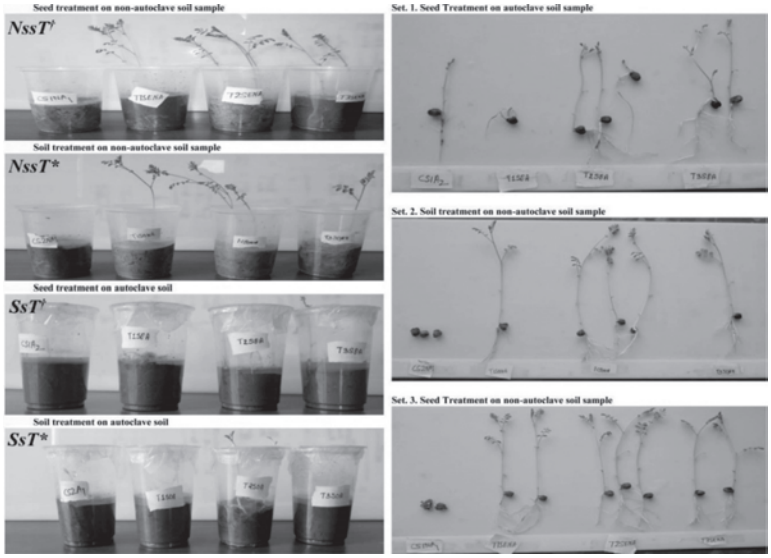

Fig. 6. *Cicer arietinum* L. growth under sterilization and non-sterilization soil by NKA1

NKA1 having a positive influence on plant in non-sterile soil with best performance.

Germination of seeds is good and also shoot-root length as well as enhanced vigour index of T1SENA: 1799.775, T2SENA: 1539.005, T3SENA: 1459.817, T1SONA: 1679.790, T2SONA: 1692.588, T3SONA: 1519.810, CS1A2: 879.890, T1SEA: 399.950, T2SEA: 1639.795, T3SEA: 1359.83, SC2A1: 479.942, T1SOA: 619.812 and T2SOA: 719.910 (Tab.2).

Root and shoot weight was also note down after the seven days of plant growing. Results showed that in non-sterile condition, CS1NA1 & CS2NA1 has no germination and plant growth without inoculants (Fig.7).

Table. 2. Vigor Index and weight of *Cicer arietinum* L. results in seven days

Treats Name	Vigor index	Root weight (gm.)			Shoot weight (gm.)		
NssT†							
CS1NA1	NG	NG			NG		
T1SENA	1799.775	0.603	0.549		0.242	0.258	
T2SENA	1539.005	0.613	0.755	0.698	0.246	0.318	0.247
T3SENA	1459.817	0.483	0.440		0.215	0.228	
NssT*							
CS2NA1	NG	NG			NG		
T1SONA	1679.790	0.545			0.303		
T2SONA	1692.588	0.610	0.550	0.481	0.324	0.232	0.264
T3SONA	1519.810	0.564			0.311		
SsT†							
CS1A2	879.890	0.626			0.088		
T1SEA	399.950	0.347			0.012		
T2SEA	1639.795	0.631			0.154		
T3SEA	1359.83	0.560	0.504	0.542	0.110	0.111	0.136
SsT*							
SC2A1	479.942	0.470			0.035		
T1SOA	619.812	0.518			0.077		
T2SOA	719.910	0.660			0.071		
T3SOA	NG	0.463	0.460	0.544	0.141	0.108	0.115

Legends: NssT†: Non-sterile + Seed treated, NssT*: Non-sterile + Soil treated, SsT†: Sterile + Seed treated, SsT*: Sterile + soil treated, NG: no growth

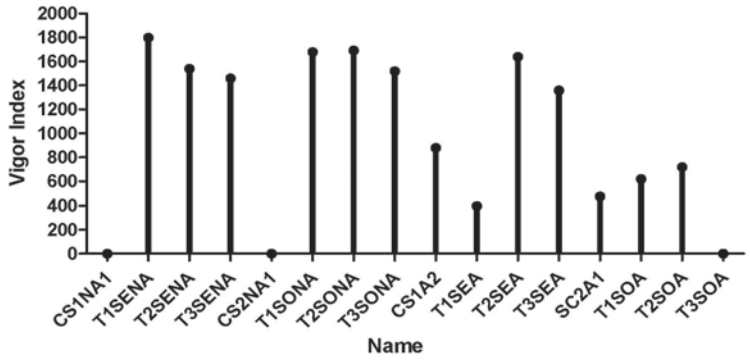

Fig. 7. Highest Vigor Index of *Cicer arietinum*

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Mean results of root (R) & shoot (S) weight of T1, 2,3SENA: < 0.591 (R) 0.276 (S); T1, 2,3SONA: < 0.550 (R) 0.283 (S); T1,2,3SEA: <0.535 (R) 0.101 (S) and T1,2,3SOA: < 0.519 (R) 0.091 (S).

The present study was focused on estimation of â-Amylase activity from *Cicer arietinum* L. (Chickpea), *Hordeumvulgare*L. (Barley) and *Phaseolus vulgaris* (Rajma) germination seeds. The seeds protein and enzyme were successfully extracted and estimated from the corresponding seeds using the principle of Lowry's method (12). For the enzyme activity, the â-amylase estimation assay was done by the adding of DNSA reagent method and standard graph (Fig.8) was plotted on the concentration of maltose.

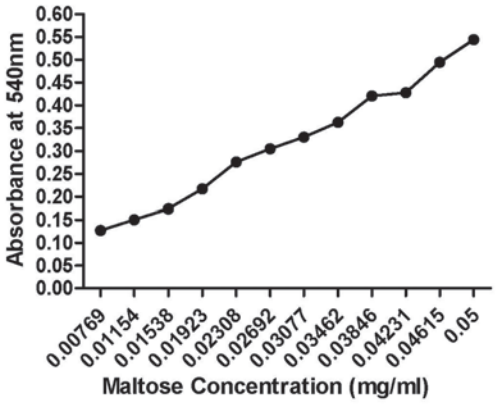

Fig. 8. Standard graph for â-Amylase estimation assay

The three crudes sample â-Amylase estimation assay absorbance result were plotted on the standard graph and result were found in the Fig. 9. CAS1E seed sample enzyme production is high in mg/mL.

The principle behind the Lowry method of determining protein concentrations of sample in the reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions and the reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential (12).Firstly, different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube series. The final volume in each of the test tubes is 5 ml. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2 ml of alkaline copper sulphate reagent (analytical reagent) and mixed the solutions well. This solution is incubated at room temperature for 10 mins. Then added 0.2 ml of reagent FolinCiocalteau solution to each tube and incubate for 30 min (23). Zero the spectrophotometer with blank and take the absorbance at 540 nm. Plot the absorbance against protein concentration to get a standard calibration curve (fig. 10).

Standard graph was finally prepared by plotting concentration of BSA in mg/ml and compared with the sample absorbance (Fig.11). HVS1P sample result showed the highest protein concentration in mg/ml.

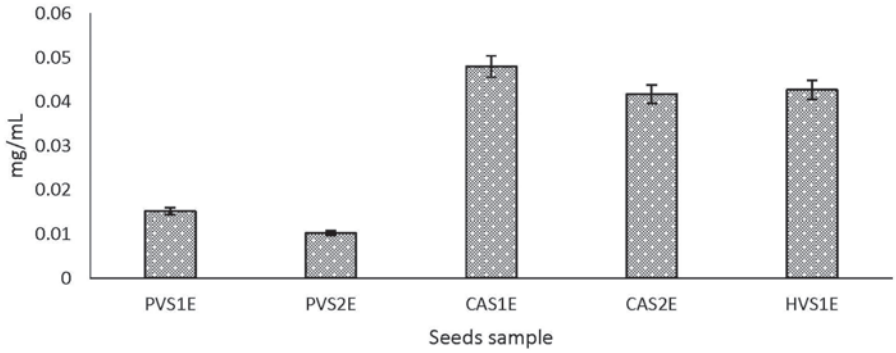

Fig. 9.â-Amylase enzyme production of *(PVS1E & PVS2E): Phaseolus vulgaris; (CAS1E & CAS2E:Cicer arietinum L.; HVS1E: Hordeumvulgare L.* in mg/mL.

The soil samples of *Menthaspicata* (MS^{1A-9A}A), *Ficusreligiosa* (FR^{1A-10A}M), *Oryza sativa* (OS^{1A-7A}D) are collected and 1gm sample added in 10ml distilled water. These samples were plated on Nutrient agar medium (HiMedia, India) for 24hrs. on 28±0.2°C. 26 bacteria isolates were screened and re-plated in culture media for morphological and biochemical characterizations results (Tab. 3).

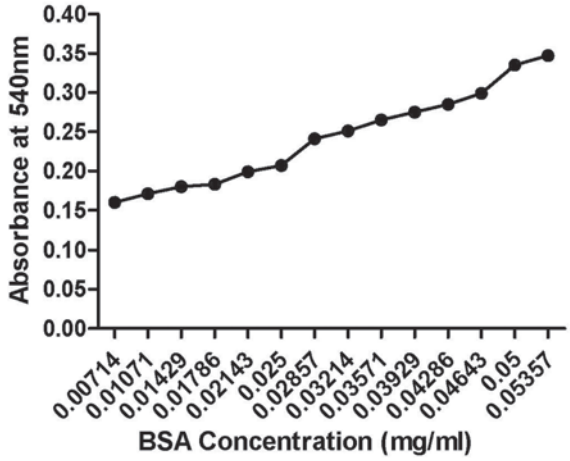

Fig. 10. Standard graph for protein concentration of the germination seeds

Spectrophotometric assay reading of á-amylase production was plotted against enzyme concentration to get a standard calibration curve and results are showed in Fig. 12. FR^{2A}M, FR^{7A}M, OS^{6A}D isolates are showed highest enzyme production to direct help in PGP.

The DNA sample of NKA1 and 14 isolates genomic DNA was run on an agarose gel (0.8%). DNA band was visualized when observed under the Gel Doc, which confirmed the purity of sample by taking A_{260} / A_{280} spectrophotometric analysis absorbance records, as the bands of DNA were single, distinct and no traces of contaminants were observed (NKA1, FR^{1A}M, FR^{2A}M, FR^{3A}M, FR^{4A}M, FR^{5A}M, FR^{6A}M, FR^{7A}M, FR^{8A}M, MS^{1A}A, MS^{2A}A & MS^{3A}A) (Fig. 13).

Conclusion

In our study, we used this NKA1 bacterium as a bio fertilizer and phosphate solubuilizer, which promote the growth of *Cicer arietinum*in non-sterile soil. Twenty six bacterial isolates were isolated from different rhizospheric soil sample. Out of all isolates, FR^{2A}M, FR^{7A}M and OS^{6A}D showed the highest PGP activity. This study suggested the rhizospheric microflora helps in plant growth promotion.

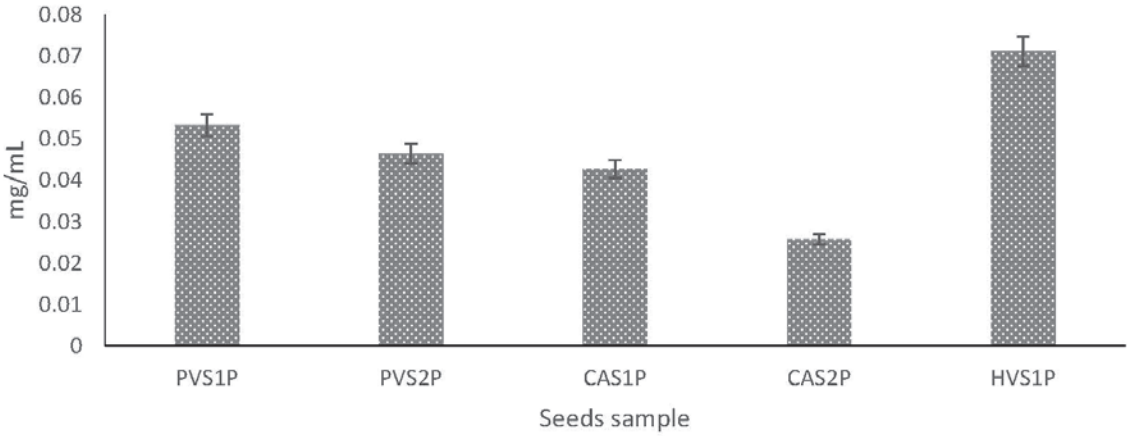

Fig. 11. Protein concentration of *(PVS1P & PVS2P): Phaseolus vulgaris; (CAS1P & CAS2P:Cicer arietinum L.; HVS1P: Hordeumvulgare L*. in mg/mL.

Plant growth promotion by bacterial inoculum with different conditions

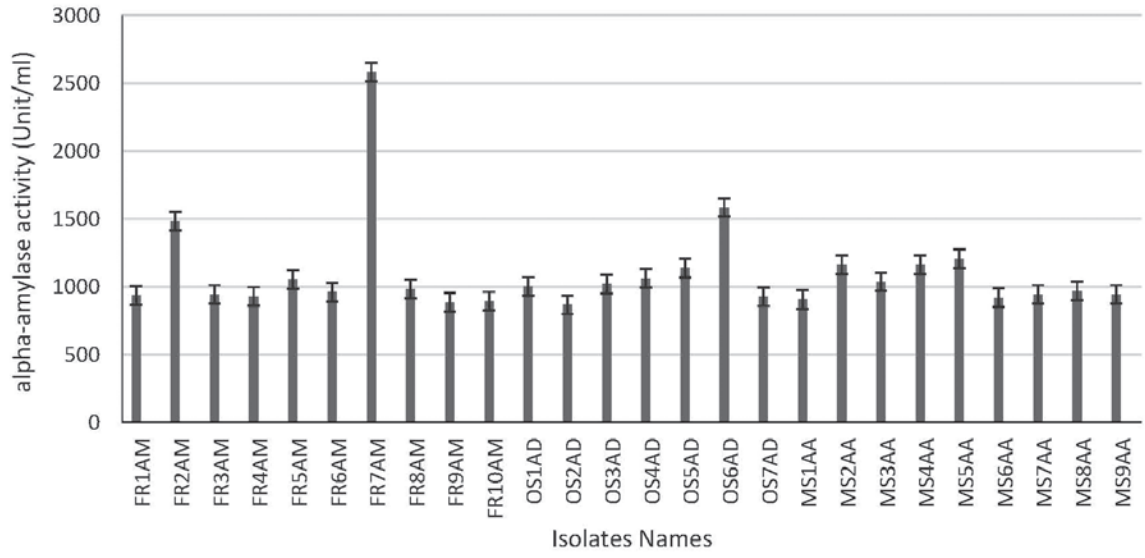

Fig. 12. Enzyme production by isolates

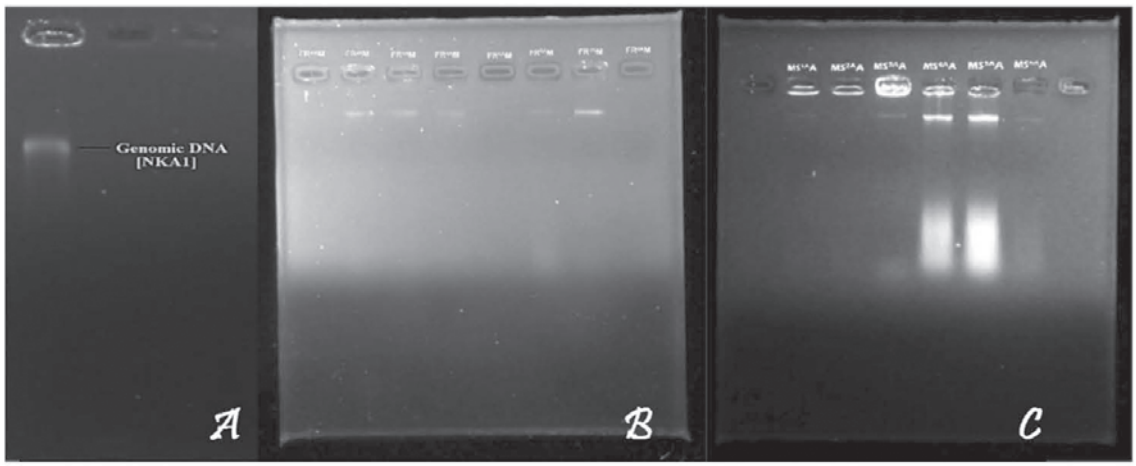

Fig. 13. Agarose gel electrophoresis showing band of genomic DNA (A: NKA1; B: $FR^{1A}M$, $FR^{2A}M$, $FR^{3A}M$, $FR^{4A}M$, $FR^{5A}M$, $FR^{6A}M$, $FR^{7A}M$ & $FR^{8A}M$; C: $MS^{1A}A$, $MS^{2A}A$, $MS^{3A}A$, $MS^{4A}A$, $MS^{5A}A$ & $MS^{6A}A$)

Table. 3. Rhizobacteria morphology and biochemical test

Isolates Name	Gram Staining	Esculine Hydrolysis	TSI	Starch Hydrolysis
$FR^{1A}M$	-	$+^{a}$	A/A,H2S	$+^{a}$
$FR^{2A}M$	+	-	K/A	$+^{c}$
$FR^{3A}M$	+	$+^{a}$	A/A	$+^{b}$
$FR^{4A}M$	-	$+^{a}$	A/A,H2S	$+^{b}$
$FR^{5A}M$	+	$+^{c}$	K/K	$+^{c}$
$FR^{6A}M$	+	$+^{a}$	K/A	-
$FR^{7A}M$	+	$+^{a}$	A/A	$+^{c}$
$FR^{8A}M$	-	$+^{c}$	K/K	$+^{c}$
$FR^{9A}M$	+	$+^{b}$	A/A	$+^{c}$
$FR^{10A}M$	+	-	A/A	$+^{c}$
$OS^{1A}D$	-	$+^{b}$	A/A	$+^{a}$
$OS^{2A}D$	+	-	K/K	$+^{c}$
$OS^{3A}D$	+	-	A/A	$+^{b}$
$OS^{4A}D$	+	-	A/A	-
$OS^{5A}D$	+	$+^{a}$	A/A	$+^{c}$
$OS^{6A}D$	+	-	A/A	-
$OS^{7A}D$	-	$+^{b}$	K/K	$+^{c}$
$MS^{1A}A$	-	$+^{b}$	A/A	$+^{c}$
$MS^{2A}A$	+	$+^{c}$	K/A	-
$MS^{3A}A$	+	-	K/A	$+^{a}$
$MS^{4A}A$	-	-	K/A	$+^{c}$
$MS^{5A}A$	+	$+^{a}$	K/A	$+^{c}$
$MS^{6A}A$	+	$+^{b}$	A/A	$+^{b}$
$MS^{7A}A$	+	$+^{a}$	K/K	-
$MS^{8A}A$	+	-	K/K	$+^{c}$
$MS^{9A}A$	+	$+^{a}$	A/A	$+^{c}$

Legends: A=Acid production, K=alkaline reaction, H2S=sulfur reaction; [a]: High, [b]: Moderate, [c]: Low; [+]: Positive, [-]: Negative.

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Bioparametric Investigation of Mutant *Bacillus subtilis* MTCC 2414 Extracellular Laccase Production under Solid State Fermentation

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Abstract

This work has been undertaken to investigate the bio parameters such as various substrates, initial moisture level, inoculum size, pH, incubation temperature, incubation period, metal ions and nitrogen sources effect on the production of laccase in solid-state fermentation using mutant *Bacillus subtilis* MTCC 2414. The laccase production was observed with a sesame oil cake (183.32 ± 0.29 U/g), initial moisture level 80% (189.28 ± 0.52 U/g), inoculum size 1.5% (196.12 ± 0.26 U/g**)**, initial pH 8 (215.20 ± 0.48 U/g), incubation temperature 37°C (225.80 ± 0.52 U/g), incubation period 48h (258.80 ± 0.29 U/g), $CuSO_4$ (263.16 ± 0.12 U/g) and yeast extract (268.14 ± 0.16 U/g) in the production medium.

Keywords: Bioparameters, solid-state fermentation, *Bacillus subtilis* MTCC 2414, laccase.

Introduction

Enzymes are delicate protein molecules ubiquitous in occurrence, and are essential for cell growth and differentiation (1, 2). The extracellular enzymes are of commercial value and find multiple applications in various industrial sectors (3). Although there are many microbial sources available for producing extracellular enzymes, only a few are recognized as commercial producers (4). Of these, strains of *Bacillus sp*. dominate the industrial sector (5). Laccase (p-diphenol: oxygen oxidoreductase; EC 1.10.3.2; also known as p-diphenol oxidase; *p-DPO;* p-diphenolase) is a copper-containing hydrolase (6), which has an ability to catalyze the oxidation of a wide variety of organic and inorganic compounds by coupling it to the reduction of oxygen to water (7). There is an increasing demand for laccase in the market for various applications such as biopulping (8), biobleaching (9), denim bleaching (10), organic synthesis (11), decolorization (12), dechlorination of xenobiotic compounds (13), bioremediation (14), plant fiber modification, ethanol production, wine stabilization, baking (15), cosmetic and dermatological preparations (16), biofuel cells *etc*. (17).

Solid state fermentation (SSF) has been known for centuries and was used successfully in the production of oriental foods (3). It has gained much importance in the production of microbial enzymes due to several economic advantages over conventional submerged fermentation (SmF) (3). Several reports on SSF have been published on the production of fine chemicals (18-22), enzymes (3, 23, 24), antibiotics (25-27), and immune suppressants (28-30). Solid state processes are of special economic interest for countries with an abundance of biomass and agro-industrial residues, as these can be used as cheap raw materials (3). Many microorganisms like species of *A.lipoferum* (31-33), *B.subtilis* (34), *B.sphaericus* (35), *B.halodurans* LBH-1 (36), *E.coli* (37), *M.mediterranea* (38), *O.iheyensis* (39), *P.maltophila* (40), *P.syringae* (41), *P.fluorescens* GB-1 (42), *P.putida* GB-1 (43), *P.desmolyticum*

NCIM 2112 (44), *P.aerophilum* (45), *Streptomycetes sp.* (46), *T.thermophilus* TTC1370 (47), *X.campesteris* (48) have been evaluated for the production of laccase.

High cost and low yields of commercial enzymes have been the main problems for its industrial production. Therefore, there is a great need to develop a new medium with inexpensive substrates that provides a high enzymes yield. Solid state fermentation has generated much interest because it reduces manufacturing cost by utilizing unprocessed or moderately processed raw materials (3, 49). There are a great number of literatures reported on the use of sawdust, coconut flesh, groundnut shell, sugarcane bagasse, banana skin, rice straw, orange bagasse, tea waste, palm oil waste, corn, wheat bran, barley as substrates for the solid state production of laccase (50, 51).

However, utilization of sunflower oil cake, sesame oil cake, cotton seed meal, apple pomace and soybean meal for the production of laccase has not been reported using the SSF. India is the largest producer of apple, cotton, soybean and rice in the world (52, 53). These relatively low priced agro-industrial residues, containing abundant nutrients (hemicellulose, cellulose, proteins and starch), have a great potential to be utilized as alternative substrates for fermentation. In the present investigation, the productivity of extracellular laccase by mutant *Bacillus subtilis* MTCC2414 using solid agro-industrial residues such as sunflower oil cake, sesame oil cake, cotton seed meal, apple pomace and soybean meal was evaluated. In addition, the culture conditions as initial moisture content, initial pH, inoculum size, incubation temperature and incubation time as well as the extra supplementation of metal ions and nitrogen sources were optimized to maximize the extracellular laccase production.

Materials and Methods

Experimental Chemicals : Sesame oil cake, sunflower oil cake, cotton seed meal, soybean meal and apple pomace were obtained from local markets at Bangalore, India. They were dried in an oven at 60°C and ground in a hammer mill (54). The ground material was passed through 30- and 50-mesh sieves (3). The fraction which passed through the 30-mesh sieve, but retained by the 50-mesh sieve was collected and used as basic fermentation media. All chemicals and reagents of analytical grade were used in this research and are mostly purchased from sigma USA and Hi media Mumbai. All the experiments were conducted in triplicate and mean values are considered.

Microorganism and Inoculum preparation : The mutant *Bacillus subtilis* MTCC 2414 strain that produces laccase was employed in the present study. The organism was mutated by UV irradiation, Ethyl methyl sulphonate (EMS) and Ethedium bromide (EtBr) for hyper production of the enzyme (2, 54-63). Stock cultures were maintained in nutrient broth medium with 70% glycerol, cultures were preserved at -20°C (64). The inoculum was prepared by transferring a loopful of stock culture (mutant *Bacillus subtilis* MTCC 2414) to a certain volume (100 ml) of sterile nutrient broth, stock medium, then incubated it overnight at 37°C on a rotary shaker 200 rpm, before being used for inoculation (2). A stock suspension was prepared and adjusted to 7×10^3 cells/ml.

Solid state fermentation : Ten grams of sesame oil cake, sunflower oil cake, cotton seed meal, soybean meal and apple pomace were taken into separate 250 ml Erlenmeyer flasks and 2 ml of salt solution [0.2% K_2HPO_4, 0.04% $CaC1_2$, 0.02% $MgSO_4$, 0.0002% $FeSO_4$, 0.001% $ZnSO_4$] was added. Distilled water was added in such a way that the final substrate moisture content was 50%. After sterilization by autoclaving, the flasks were cooled to room temperature and inoculated with 1% (v/w) inoculum of 24h culture and incubated at 37°C for 48h.

Estimation of different moisture contents : The moisture content of the substrates was estimated by drying 10 g of substrates to constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, the substrates were soaked with desired quantity of

additional water (3, 65). After soaking, samples were dried again as described above and percent of moisture content was calculated as follows,

Percent of moisture content (initial) of solid medium =

$$\frac{\text{(Wt. of the Substrate - Dry wt. of Substrate)}}{\text{Dry wt. of Substrate}} \times 100$$

Measurement of pH : The pH was determined using 1g of fermented material in 10ml of distilled water, and then the mixture was agitated. After 10min, the pH was measured in the supernatant using a pH meter (3).

Determination of Laccase Activity : Laccase activity was measured by monitoring the oxidation of 1mM guaiacol (Hi media, Mumbai, India) buffered with 0.2 M sodium phosphate buffer (pH 6) at 420 nm for 1 min. The reaction mixture (900 µl) contained 300 µl of 1 mM guaiacol, 300 µl of culture filtrate, and 300 µl of 0.2 M sodium phosphate buffer (pH 6). One unit of the enzyme activity was defined as the amount of enzyme that oxidized 1 µmol of guaiacol per minute. The enzyme activity was expressed in U/ml (66).

Bioparametric Investigation on Extracellular Laccase Production under Solid State Fermentation

Effect of different solid substrates : In SSF, the solid substrate not only serves as an anchor, but also supplies nutrients to the organism. The chemical composition of the substrate is of critical importance. The ideal solid substrate is the one that provides maximum nutrients to the microorganism for its optimal growth and metabolic function. The solid substrates, sunflower oil cake, sesame oil cake, apple pomace, cotton seed meal and soybean meal have been identified as ideal substrates and used to study their effect on the production of extracellular laccase. Each substrate (10g) was taken into a separate 250 ml Erlenmeyer flask and 2 ml of salt solution [0.2% K_2HPO_4, 0.04% $CaC1_2$, 0.02% $MgSO_4$, 0.0002% $FeSO_4$, 0.001% $ZnSO_4$] was added. Distilled water was added in such a way that the final substrate moisture content was 50%. After sterilization by autoclaving, the flasks were cooled to room temperature and inoculated with 1% (v/w) inoculum of 24h culture and incubated at 37°C for 48h (3). The samples were assayed as per above mentioned protocol (66). The best solid substrate achieved by this step for the maximum production of laccase was fixed for the subsequent experiments.

Effect of different initial moisture contents : To investigate the influence of the initial total moisture content of the substrate (sesame oil cake), the fermentation was carried out under various initial moisture contents (20, 30, 40, 50, 60, 70, 80, 90 and 100%) of substrate, which was adjusted with distilled water (3). The other fermentation conditions were sesame oil cake as substrate, 1% inoculum level, 2ml salt solution and the fermentation was carried out for 48h at 37°C. The samples were assayed as per above mentioned protocol (66). The optimum initial moisture content of solid substrate achieved by this step for maximum laccase production was fixed in subsequent experiments.

Effect of different inoculum levels : Various inoculum levels were tried (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5%) to study their effect on laccase production (3). The fermentation was carried out using sesame oil cake as substrate. The other conditions were moisture content 80%, salt solution, 2ml, incubation temperature 37°C and incubation period 48h. The samples were assayed as described earlier (66). The optimum inoculum level achieved by this step was fixed in subsequent experiments.

Effect of different initial pH : pH varying from 3.0 to 11.0 (adjusted with 1N HCl or 1N NaOH) was used to optimize initial pH of the basal media (3). The fermentation was carried out at 37°C to study their effect on laccase production. The other conditions were sesame oil cake as substrate, 1.5% inoculum level, moisture content of 80%, salt solution 2ml, incubation temperature 37°C and the fermentation time of 48h. The samples were assayed as described earlier (66). The optimum

pH achieved by this step was fixed in subsequent experiments.

Effect of different incubation temperatures : The fermentation was carried out at various temperatures such as 20, 25, 30, 37, 40, 45, 50°C (3) to study their effect on laccase production. The fermentation was carried out using the above optimized conditions viz., sesame oil cake as substrate, moisture content 80%, salt solution 2ml, pH8 and inoculum level 1.5% for 48h. The samples were assayed as described earlier (66). The optimal incubation temperature obtained in this step was used in the subsequent experiments.

Effect of different incubation periods : Different incubation periods (48, 72, 96, 120, 144, 168, 192, 216 and 240h) were employed to study their effect on laccase production (3). The fermentation was run using the above optimized conditions viz., substrate sesame oil cake, moisture content 80%, salt solution 2ml, inoculum level 1.5%, pH8 and incubation temperature of 37°C. The samples were assayed as described earlier (66). The optimum incubation period achieved by this step was fixed in subsequent experiments.

Effect of different metal ions and inorganic nitrogen sources : Different metal ions and inorganic nitrogen sources (0.5%) K_2HPO_4, $CaC1_2$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, KNO_3, NH_4Cl, $(NH_4)_2HPO_4$, NH_4NO_3, $CuSO_4$ and $(NH_4)_2SO_4$) were employed to study their effect on laccase production (3). The fermentation was carried out using the above optimized conditions viz., substrate sesame oil cake, moisture content 80%, salt solution 2ml, inoculum level 1.5%, pH8, incubation period 48h and incubation temperature of 37°C. The samples were assayed as described earlier (66). The optimum enzyme production was achieved by this step was fixed in subsequent experiments.

Effect of different organic nitrogen sources : Different organic nitrogen sources (Casein, Yeast extract, Peptone, Serine, Histidine and Aspartate 1%) were employed to study their effect on laccase production (3). The fermentation was carried out using the above optimized conditions viz., substrate sesame oil cake, moisture content 80%, salt solution 2ml, inoculum level 1.5%, $CuSO_4$ (0.5%), incubation period 48h, pH8 and incubation temperature of 37°C. The samples were assayed as described earlier (66). The optimum enzyme production was achieved by this step was fixed in subsequent experiments.

Results and Discussion

Evaluation of different agro-industrial material for extracellular laccase production : The fermentation profile of laccase production in SSF varied with the type of agro material used. Highest laccase production (183.32 ± 0.29 U/g substrate) was observed with a sesame oil cake and the least laccase production (170 ± 0.32 U/g substrate) was observed with sunflower oil cake (Fig. 1). Variation was noticed with these materials. This could be attributed to solid materials dual role-supply of nutrients and anchor to the growing microbial culture which influence the microbial growth and subsequent metabolite production. Such substrate dependent microbial product yield variations were also reported in the literature. These results depict that the selection of an ideal agro biotech source for laccase production depends primarily on the availability of carbon and nitrogen source and thus screening of several agro-industrial residues are essential.

However, in the present study, among all studied materials, sunflower oil cake supported least production of laccase. This may be attributed to the fact that the strains used may vary in their

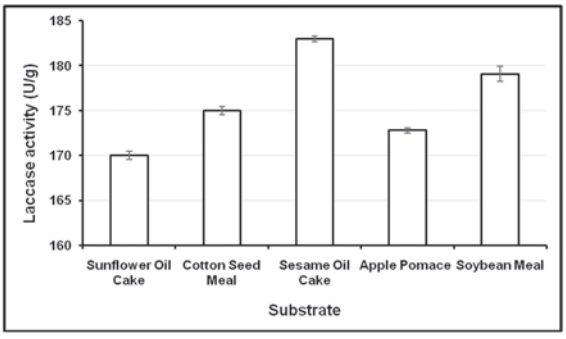

Fig: 1. Effect of various substrates on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

metabolic pattern compared to *Bacillus subtilis* MTCC 2414 used in the present study or carbon source material associated with sunflower oil may not be utilized by the *Bacillus subtilis* MTCC 2414. To evaluate the same, the hemicellulose and cellulose hydrolysis ability of the strain was investigated. This data further confirmed that high laccase associated with sesame oil cake were due to the maximum production of hemicellulose and cellulose hydrolyzing enzyme by the strain and as strain *Bacillus subtilis* MTCC 2414 is hemicellulase positive hence could utilize hemicellulose as carbon source. Hence, it could be concluded that the selected strain requires substrates that provide hemicellulose as its enzymatic machinery that hydrolyzes the polysaccharides present in substrates.

Effect of moisture level : The moisture level in the solid-state fermentation critically affects the process due to its interference in the physical properties of the solid particle. Increased moisture is believed to reduce the porosity of substrate, thus limiting the oxygen transfer. The decreased moisture content causes lower availability of media nutrients to the *Bacillus subtilis* MTCC 2414 resulting into the lower extent of production. The effect of total moisture content on laccase production for 20, 30, 40, 50, 60, 70, 80, 90 and 100% moisture was investigated. The result indicated that 80% moisture gave the higher laccase production during fermentation compared to other treatments. The maximum yield of laccase production (189.28 ± 0.52 U/g substrate) was obtained from 80% moisture (Fig. 2). The results from the previous study stated that the reduction in enzyme yield could occur with low and to higher moisture level.

Effect of inoculum size : The optimum inoculum size for laccase production (196.12 ± 0.26 U/g substrate) by *Bacillus subtilis* MTCC 2414 was 1.5 $\times 10^6$ CFU/g initial dry substrate (Fig. 3). Adequate inoculum can initiate fast growth and product formation, thereby reducing the growth of contaminants. A decrease in enzyme production was observed when the inoculums size was increased beyond the optimum level. Enzyme production attains its peak when sufficient nutrients are available for the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased laccase synthesis.

Effect of initial pH of the medium on laccase production : The initial pH of the fermentation media may change during fermentation because the substrates employed in SSF usually have the least buffering. Some samples from the fermented mass were aseptically withdrawn, homogenized and pH was checked. The pH of the medium during fermentation was found to be between 3.0 and 11.0, i.e. around acidic to alkaline conditions. The initial pH is another important factor which affects the growth and enzyme production during solid-state fermentation. The substrate was adjusted to different initial pH using 1N HCl and 1N NaOH prior

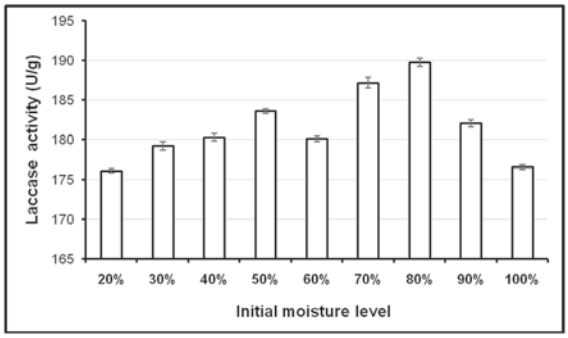

Fig. 2: Effect of various moisture level (%) on laccase production by *Bacillus subtilis* MTCC 2414under SSF

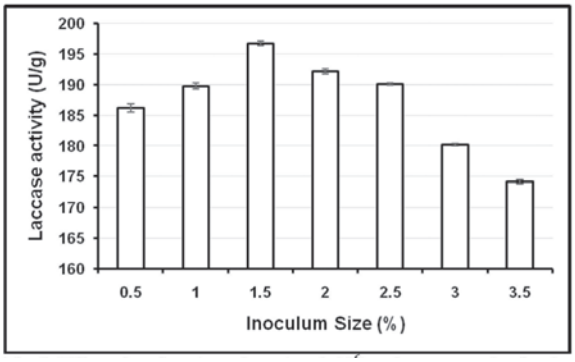

Fig. 3: Effect of various inoculum sizes ($\times 10^6$) on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

to inoculation. The maximum yield of laccase production (215.20 ± 0.48 U/g substrate) was observed in an initial pH of 8.0 (Fig. 4).

Effect of temperature : The maintenance of an optimal process temperature is one of the major factors in the economics of a process. Temperature affects microbial cellular growth, spore formation, germination and microbial physiology, thus affecting product formation in turn. 37°C was found to be the optimum temperature in this case. The maximum yield of laccase production (225.80 ± 0.52 U/g substrate) was observed at 37°C (Fig. 5).

Effect of incubation period : Solid-state process was performed for various incubation periods. Remarkably maximal level (258.80 ± 0.29 U/g substrate) of laccase was achieved after 48h of fermentation (Fig. 6). Important rise in laccase yield with increased biomass was observed during 48 - 72h of fermentation cycle. Significant variation in laccase production was observed during different fermentation periods.

Effect of metal ions and nitrogen sources on laccase production : Generally, the high concentration of metal ions and nitrogen sources in media is effective in enhancing the production of laccase by *Bacillus subtilis* MTCC 2414. The protein content in sesame oil cake is very low so that the nitrogen levels as well as the commercial value decrease greatly. Hence, the exogenous addition of various metal ions, inorganic and organic nitrogen levels to the solid medium was studied.

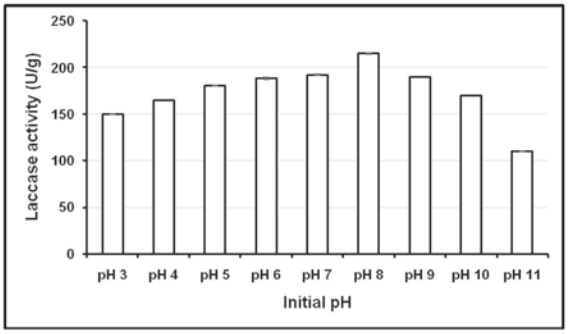

Fig. 4: Effect of various initial pH on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

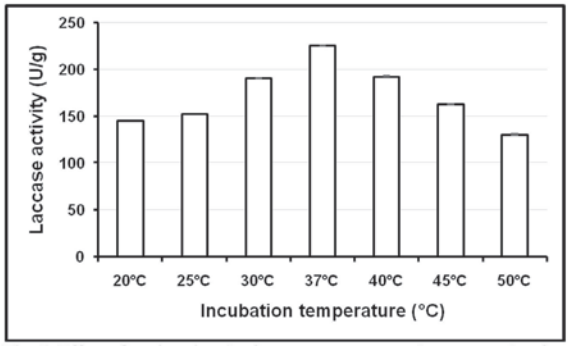

Fig. 5: Effect of various incubation temperatures on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

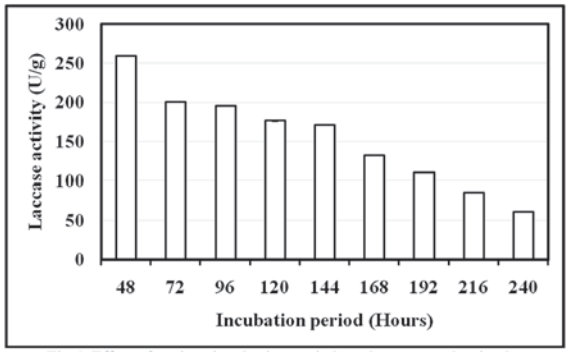

Fig.6: Effect of various incubation periods on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

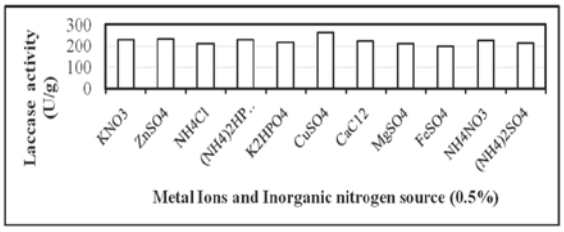

Fig. 7: Effect of various metal ions and inorganic nitrogen sources (0.5 %w/v) on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

$CuSO_4$, $CaC1_2$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, KNO_3 and K_2HPO_4 as metal ions, NH_4Cl, $(NH_4)_2HPO_4$, NH_4NO_3 and $(NH_4)_2SO_4$ as inorganic nitrogen sources and casein, yeast extract, peptone, serine, histidine and aspartate were used as complex organic nitrogen sources. Based on the results it was found that $CuSO_4$ was the best metal ion source (263.16

± 0.12 U/g substrate) and yeast extract was the best organic nitrogen source (268.14 ± 0.16 U/g substrate) its supplementation led to further increase in laccase production (Fig. 7 and 8).

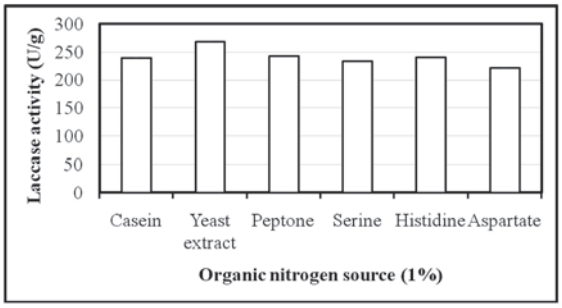

Fig. 8: Effect of various organic nitrogen sources (1 %w/v) on laccase production by *Bacillus subtilis* MTCC 2414 under SSF

Conclusion

The role of agro wastes in laccase production was identified and production parameters were determined. The sesame oil cake can be the least expensive alternative active substrates in the production of laccase. The optimal conditions for laccase production using SSF for sesame oil cake were initial pH (8), initial moisture level (80%), inoculum size (1.5 x 10^6 CFU/g), incubation temperature (37°C), incubation period (48h), $CuSO_4$ (0.5% w/v), yeast extract (1% w/v) respectively. Such processes would not only help in reducing the cost of production, but also pave the way in effective solid waste management. With the above encouraging leads, it will be interesting to study the sesame oil cake as substrate for the production of other enzymes from different microbes.

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Formulation and Evaluation of Etodolac Oral Disintegrating Tablets

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Abstract

Etodolac is a nonsteroidal anti-inflammatory drug, which result in inhibition of the enzyme cyclooxygenase (COX). The aim of this study is to formulate and evaluate oral disintegrating tablets (ODTs) of etodolac to achieve rapid dissolution, absorption and further improving the bioavailability of the drug. The oral disintegrating tablets were prepared by using Croscarmellose sodium, Sodium starch glycolate and Crospovidone by direct compression method. Taste masking was done by flavouring agents. Drug-polymer complex was then formulated into orally disintegrating tablets by direct compression by using different concentrations of superdisintegrants. Tablets were evaluated for weight variation, thickness, hardness, friability, drug content, *in vitro* disintegration time, wetting time, water absorption ratio, and *in vitro* dissolution studies. Total nine formulations were prepared (i.e. F1 to F9), out of which tablets with F9 formulation containing 9% crospovidone showed faster disintegration within 15.05 seconds.

Keywords: Superdisintegrants, etodolac, anti-inflammatory, and flavouring agents**.**

Introduction

Among the available pharmaceutical dosage forms, tablets are the most widely used dosage form because of their convenience in terms of self-medication, ease of administration, accurate dosage, compactness, good stability and ease of manufacturing. The elderly people would experience deterioration of their physiological and physical abilities like dysphagia (difficulty in swallowing). Pediatric patients may suffer from ingestion problems of their underdeveloped muscular and nervous system (1). In order to overcome this problem, a new drug delivery system has been developed known as Orally Disintegrating Tablets (ODTs). Orally Disintegrating Tablets are solid dosage form containing medicinal substances which disintegrates/dissolves rapidly upon contact with saliva. When these tablets are placed in the oral cavity, saliva penetrates into the pores causing rapid disintegration. These tablets are beneficial for the patients suffering from nausea and vomiting, those with mental disorders, bedridden and those who do not have easy access to water.

The U.S. Food and Drug Administration Center for Drug Evaluation and Research (CDER) defines, in the 'Orange Book', an ODT as "a solid dosage form containing medicinal substances, which disintegrates rapidly, usually within a matter of seconds, when placed upon the tongue".

Recently, European Pharmacopoeia used the term 'Orodispersible tablet' as a tablet that is to be placed in the mouth where it disperses rapidly before swallowing.

Orally disintegrating tablets are also called as mouth-dissolving tablets, fast disintegrating tablets, fast dissolving tablets, orodispersible tablets, rapi-melts, porous tablets, and quick dissolving tablet (2).

Materials and methodology

Materials

Etodolac was received as a gift sample from IPCA Laboratories Ltd., Mumbai. Sodium starch glycolate was procured from LOBA Chemie

Laboratory Reagents and Fine Chemicals, Mumbai. Croscarmellose sodium and crospovidone were procured as gift samples from Ranbaxy Laboratories Limited, Gurugram, India. Mannitol was procured Finar Chemicals Limited, Ahmedabad, India. Talc and magnesium stearate were purchased from the S D Fine-Chem Ltd., Mumbai. All other chemicals were of analytical grades.

Preformulation studies

Drug-excipient compatibility studies

Fourier Transform-Infrared Spectroscopic Studies : A Fourier Transform–Infrared Spectrophotometer (FTIR) was used to study the non-thermal analysis of drug-excipient (the binary mixture of drug: excipient 1:1 ratio) compatibility. Pure drug of Etodolac and drug with a physical mixture of F9 formulation (excipients) compatibility studies were performed. The spectrum of each sample was recorded over 100-4000 cm^{-1}.

Final Powder Blend : The powder blend of all formulations was evaluated for Bulk density, Tapped density, Compressibility index, Hausner ratio and Angle of repose.

A) Bulk Density : A 30 g of material was passed through a sieve no.25 to break up agglomerates and introduced into a dry 100 ml cylinder, without compacting, the powder was carefully levelled without compacting and the unsettled apparent volume (Vo) was read. The bulk density was calculated, in grams per ml, using the formula:

$$\text{Bulk Density} = (M) / (V_O)$$

Where

M = Total weight of the powder blend; V_0 = Bulk volume of the powder blend

B) Tapped Density *:* After carrying out the procedure as given in the measurement of bulk density, the cylinder containing the sample was tapped using a mechanical tapped density tester (Electrolab) that provides a fixed drop of 14±2 mm at a nominal rate of 300 drops per minute. The cylinder was tapped 500 times initially followed by an additional tap of 750 times until difference between succeeding measurement was less than 2% and then tapped volume (V_f) was measured to the nearest graduated unit. The tapped density was calculated, in grams per ml, using the formula:

$$\text{Tapped Density} = (M) / (V_f)$$

Where

M = Total weight of the powder blend; V_f = Tapped volume of the powder blend

C) Measures of Powder Compressibility : The Compressibility Index and the Hausner Ratio are measures of the propensity of a powder to be compressed. As such, they are measures of the relative importance of inter particulate interactions. In a free-flowing powder, such interactions are generally less significant and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater interparticle interactions and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the Compressibility Index and the Hausner Ratio, which are calculated using the following formula:

$$\text{Compressibility Index} = (Vr\text{-}Vo) * 100 / Vr$$

Where

Vr = Tapped density; Vo = Bulk density

D) Hausner Ratio : It is the ratio of bulk density to tapped density.

$$\text{Hausner Ratio} = Vo/Vr$$

Where

Vo = Bulk density; Vr = Tapped density

E) Angle of Repose : The fixed funnel method was employed to measure the repose angle. A funnel was secured with its tip at a given height (h) above a graph paper that was placed on a flat horizontal surface. The blend was carefully poured through the funnel until the apex of the conical pile just touched the tip of the funnel. The radius (r) of the base of the conical pile was measured. The angle of repose (θ) was calculated using the following formula:

$$\text{Angle of Repose } (\theta) = \tan^{-}1(h/r)$$

Where

h = height of pile; r = radius of the base of the pile; θ = angle of repose

Analytical method used in the determination of etodolac

Preparation of standard curve for etodolac : A standard stock solution of pure drug containing 100 mg of Etodolac/100 ml was prepared using 6.8 pH phosphate buffer. The working standards were obtained by dilution of the stock solution. The standard curves for Etodolac were prepared in a concentration range of 2-12 µg/ml at the selected wavelength of 225 nm. Their absorptivity values were used to determine the linearity. The solution was scanned and Beer-Lambert Law limit was obeyed in the concentration range of 2, 4, 6, 8, 10 and 12 µg/ml (figure 1).

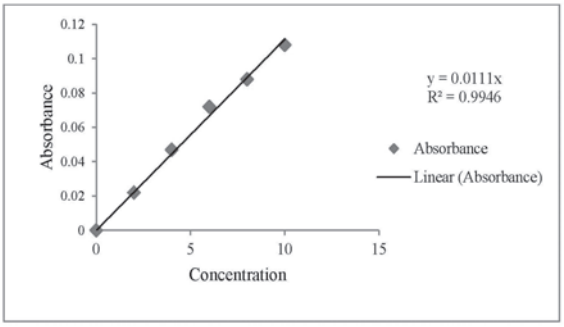

Fig. 1. Calibration curve of Etodolac

Formulation Design : Etodolac ODTs were prepared using direct compression technique. Different formulations of ODTs were designed to be prepared by direct compression technique using three super disintegrating agents (Crospovidone, Croscarmellose sodium and Sodium starch glycolate). Super disintegrating agents are varied with three different concentrations of 3, 6 and 9% respectively. Keeping all other ingredients constant, they are assigned with formulation codes in table 1.

Preparation Method : All the required ingredients were passed through sieve no. 40 to get uniform size particles and weighed accurately. The whole amount of Etodolac drug, sodium starch glycolate or croscarmellose or crospovidone, talc and flavour except lubricant were mixed in the increasing order of their weights in a mortar. To this mixture, talc and magnesium stearate were added. The final mixture was shaken manually for 5-10 minutes in a plastic bag. This powder was passed through the hopper of 16 station rotary tableting machine and punched into tablets using 9 mm s/c. The process is similar for all the formulations, which are prepared by direct compression technique.

Different quality control tests were performed for all the ODT formulations to check whether they have met the specifications given in USP along with other *in vitro* tests like wetting time and water absorption ratio.

Evaluation Parameters

- Weight variation test
- Thickness measurement
- Hardness and Friability
- Drug content uniformity
- Disintegration time
- Wetting time and Water absorption ratio
- *In vitro* dispersion time
- Dissolution test

Weight variation test : Twenty tablets were randomly selected from each formulation and their average weight was calculated by using an Electronic Balance (Shimadzu, AUX 220, Shimadzu Corp, Japan). Individual weight of each tablet was also calculated using the same and compared with the average weight. The mean ± S.D. was noted.

Thickness measurement : Randomly ten tablets were taken from each formulation and their thickness was measured using a Digital Vernier Caliper (Mitutoyo Corp, Kawasaki, Japan). Average thickness and standard deviation values were calculated. The tablet thickness should be controlled within a ± 5% variation of standard value.

Hardness : The tablet hardness of different formulations was measured using the Monsanto Hardness Tester. The tester consists of a barrel containing a compressible spring held between two plungers. The lower plunger was placed in contact with the tablet and a zero was taken. The upper plunger was then forced against the spring by turning a threaded bolt until the tablet fractures.

As the spring is compressed, a pointer rides along a gauge in the barrel to indicate the force. The force of fracture is recorded and the zero force reading is deducted from it. Generally, a minimum hardness of 4 kg is considered acceptable for uncoated tablets. The hardness for ODTs should be preferably 1-3 kg.

Friability : This test is performed using a laboratory friability tester known as Roche Friabilator. Ten tablets were weighed and placed in a plastic chambered friabilator attached to a motor, which revolves at a speed of 25 rpm (rotations per minute) dropping the tablets from a distance of 6 inches with each revolution. The tablets were subjected to 100 revolutions for 4 minutes. After the process, these tablets were de-dusted and reweighed. Percentage loss of tablet weight was calculated:

$$\% \text{ Friability} = (W_1 - W_2) \times 100/W_1$$

Where

W_1 = Initial weight of the 10 tablets; W_2 = Final weight of the 10 tablets.

Drug content uniformity : Ten tablets were randomly selected, weighed and finely powdered and quantity of powder equivalent to one tablet was added to 100 ml of 6.8 pH phosphate buffer in a conical flask. Conical flasks were placed on a rotary shaker. An aliquot of solution was centrifuged and supernatant was filtered through a 0.22 µ filter. The absorbance of the resulted supernatant solution was measured using UV-Visible Spectrophotometer at a wavelength of 225 nm against 6.8 pH phosphate buffer as blank. Concentrations were calculated with the help of standard graph and the total amount present in the formulation was calculated.

Disintegration time : Disintegration time is considered to be one of the important criteria in selecting the best formulation. *In vitro* disintegration time for ODTs was determined by using USP disintegration apparatus (Electrolab ED-2L, India) with 6.8 pH phosphate buffer as the disintegration medium. The medium was maintained at 37±0.5ºC. The time point at which tablet completely disintegrates is noted as disintegration time.

Wetting time : A piece of tissue paper folded twice was placed in a small petri dish containing 6 ml of water. A water-soluble dye phenolphthalein was added to the petri dish. The dye solution is used to identify the complete wetting of the tablet surface (4). A tablet was carefully placed on the surface of tissue paper in the petri dish at room temperature. The time required for water to reach the upper surface of the tablets and completely wet them was noted as the wetting time. To check for reproducibility, the measurements were carried out in replicates (n=6). The wetting time was recorded using a stopwatch.

Water absorption ratio (R) : The weight of the tablet before keeping in the petri dish was noted (W_b) using Shimadzu Digital Balance. The wetted tablet from the petri dish was taken and reweighed (W_a) using the same. The Water absorption ratio (R) was determined according to the following equation:

$$R = 100 (W_a - W_b) / W_b$$

Where

W_b = Weight before water absorption;
W_a = Weight after water absorption.

***In vitro* dispersion time :** *In vitro* dispersion time was measured by dropping a tablet in a beaker containing 100 ml of 6.8 pH phosphate buffer. Three tablets from each formulation were randomly selected and *in vitro* dispersion time was performed.

Dissolution test : The dissolution test was carried out using USP apparatus 2 (Paddle type). The stirring rate was 50 rpm. A 6.8 pH phosphate buffer and methanol (1:1) was used as dissolution medium (900 ml) and was maintained at 37±1^{0}C. Samples of 5 ml were withdrawn at predetermined intervals (2, 4, 6, 8, 10, 15, 20, 25 and 30 min), filtered and replaced with 5 ml of fresh dissolution medium. The collected samples were suitably diluted with dissolution fluid wherever necessary and were analyzed for the Etodoloc at 225 nm by using UV spectrophotometer. Each dissolution study was performed for three times and mean values were taken.

Results and Discussion

Oral disintegrating tablets of Etodolac were prepared by direct compression method using Crospovidone (CP), Croscarmellose sodium (CCS), and Sodium Starch Glycolate (SSG) as superdisintegrants. A total of 9 formulations (F1 to F9) were designed.

Construction of Calibration Curve : The study started with the construction of standard calibration curve. The λ_{max} of Etodolac in 6.8 pH phosphate buffer was scanned and found to have the maximum absorbance at 225 nm. The standard graph of Etodolac in 6.8 pH phosphate buffer was plotted by taking concentration ranging from 2-12 µg/ml and a good correlation was obtained with R^2 values of 0.994 respectively.

Precompression Parameters : The values of precompression parameters evaluated were found to be within the prescribed limits and indicated good free flowing property (table 3). Flow properties of the powder and resistance to the particle movement can be judged from the angle of repose. The values for an Angle of repose were found to be in the range of 28.12 to 30.35. The Carr's index of the prepared blends falls in the range of 16.26% to 19.08% and this is also supported by the Hausner's ratio values, which were in the range of 1.19±0.16 to 1.28±0.23. Hence, the prepared blends possessed good flow properties and these blends can be used for tablet manufacture.

Weight Variation Test : All the tablets were prepared under similar conditions. All the formulations exhibited white colour and odorless with a smooth surface. The average weight of the ODTs prepared by direct compression method was 291.12±0.39 to 300.01±0.09 mg.

Thickness Measurement : The thickness of all the formulations was within acceptable limits. The thickness of tablets was measured using a Digital Vernier Caliper. The thickness of tablets was found in between 3.05±0.10 to 3.15±0.09.

Hardness : The hardness of all the formulations was within acceptable limits. The hardness of tablets prepared by direct compression was 2.78±0.23 to 2.98± 0.39 kg/cm^2.

Friability : The friability of all formulations was found to be 0.41 to 0.68 and hence the tablets with lower friability may not break during handling on machines and or shipping. Various evaluated parameters for an oral disintegrating tablet formulation of Etodolac are shown in table 3 & 4.

Wetting Time : Wetting time of all the formulations was in the range of 08.39 to 25.02 seconds. ODTs of F9 formulation showed the least (08.39 sec) wetting time.

Water Absorption : Water absorption ratio of all the formulations was in the range of 12.77 to 30.56%. ODTs of F9 formulation showed the least (12.77%) water absorption ratio.

In vitro dispersion time: *In vitro* dispersion time of all the formulations were in the range of 18 to 38 seconds. ODTs of F9 formulation showed the least (18.56 sec) dispersion time.

Disintegration time*:* Disintegration time is very important for ODTs, which is desired to be less than 60 seconds for orally disintegrating tablets. This rapid disintegration assists swallowing and also plays a role in drug absorption in the buccal cavity, thus promoting bioavailability. The disintegration time of prepared ODTs was in the range of 15.05 to 32.41 seconds and the order is CP < SSG < CCS. Among all the formulations, the F9 formulation was prepared with Crospovidone in the concentration of 9% as disintegrant exhibit least disintegration time (15.05 sec). As the concentration of superdisintegrants in the formulations increased, the disintegration time was found to decrease.

Drug Content Uniformity : The drug content of all the formulations (F1 to F9) was found to be between 85.48 and 99.09%, which was within the acceptable limits. For most of the larger dose drugs in tablet form, the official potency range permitted is not less than 95% and not more than 105% of the labelled amount.

Dissolution Test : *In vitro* dissolution data of all the Etodolac ODT formulations are shown in table 5 and corresponding dissolution profile is shown in figure 2, 3 & 4. The F9 formulation exhibits the better dissolution profile than that of all Etodolac ODT formulations.

Drug-Excipient Compatibility Studies : The compatibility between the drug, polymer and excipients was compared by FTIR spectroscopy (Perkin Elmer). The FTIR spectrum of pure drug exhibits characteristic peaks at 3350, 1397, 889 and 2966 cm^{-1} due to N-H, C-O, C-N and C-H stretching respectively. The FTIR spectrum of optimized formulation (F9) showed characteristic peaks at 3351, 1422, 1042 and 2915 cm^{-1}. The presence of above peaks confirm undisturbed drug in the formulations. It was observed that there was no disappearance or shift in peak position of the drug in any spectra of drug and superdisintegrants, which proved that drug and superdisintegrants were compatible. The FTIR data interpretation of drug and optimized formulation was shown in table 6.

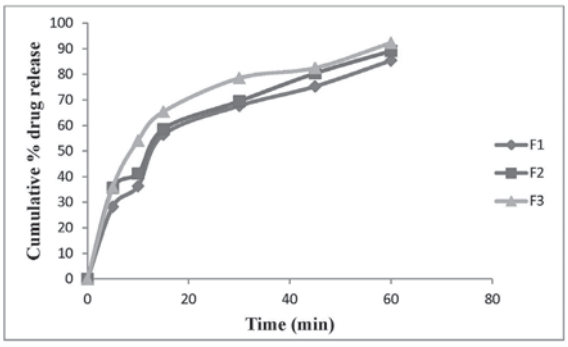

Fig. 2. Cumulative % drug release of Etodolac ODTs prepared individually varying concentrations of Superdisintegrating agents

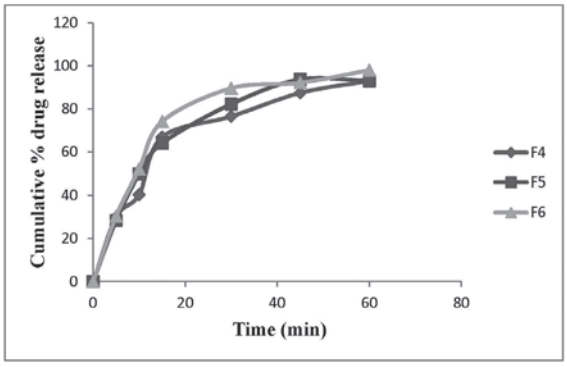

Fig. 3. Cumulative % drug release of Etodolac ODTs prepared individually varying concentrations of super disintegrating agents

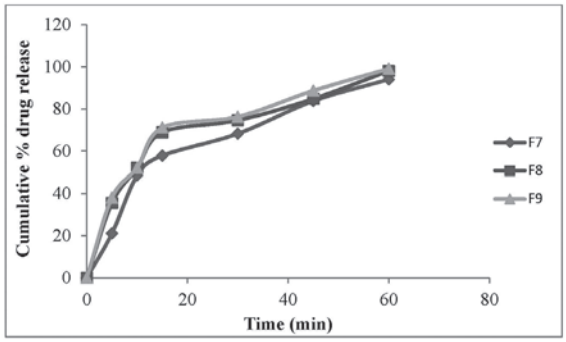

Fig. 4. Cumulative % drug release of Etodolac ODTs prepared individually varying concentrations of Superdisintegrating agents

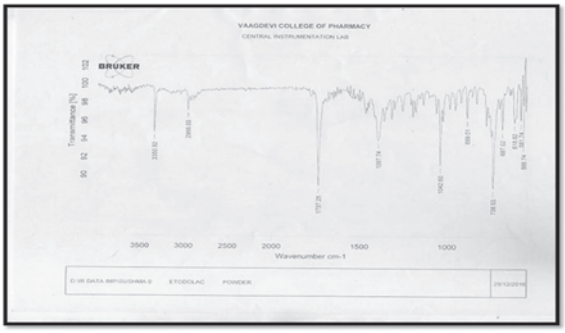

Fig. 5: FTIR studies of drug Etodolac

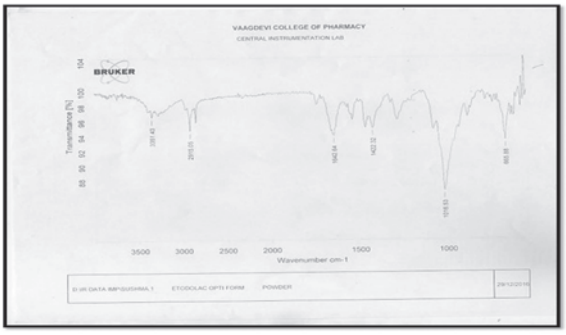

Fig. 6. FTIR studies of optimized formulation (F9)

Table 1. Formulation of Etodolac ODTs

Disintegrating Agents	Concentration (%)	Formulation Code
Sodium starch glycolate	3	F1
	6	F2
	9	F3
Croscarmellose sodium	3	F4
	6	F5
	9	F6
Crospovidone	3	F7
	6	F8
	9	F9

Table 2. Formula of ODTs Prepared by Direct Compression Method using Super Disintegrants Individually

Ingredients (mg)	Formulation Code								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Etodolac	200	200	200	200	200	200	200	200	200
Sodium starch glycolate	3	6	9	-	-	-	-	-	-
Croscarmellose sodium	-	-	-	3	6	9	-	-	-
Crospovidone	-	-	-	-	-	-	3	6	9
Mannitol	30	30	30	30	30	30	30	30	30
Talc	1	1	1	1	1	1	1	1	1
Magnesium stearate	1	1	1	1	1	1	1	1	1

Table 3. Preformulation Characteristics of Etodolac ODTs

Formulation Code	Bulk Density (g/c)	Tapped Density (g/cc)	Hausner's Ratio	Compressibility Index (%)	Angle of Repose
F1	0.425±0.15	0.527±0.25	1.19±0.16	16.75	28.98
F2	0.428±0.35	0.514±0.27	1.20±0.30	17.37	30.21
F3	0.429±0.22	0.521±0.29	1.28±0.22	18.46	30.19
F4	0.413±0.16	0.512±0.21	1.25±0.16	17.60	28.43
F5	0.417±0.37	0.515±0.28	1.22±0.26	18.71	30.35
F6	0.433±0.16	0.509±0.24	1.20±0.21	16.26	30.20
F7	0.423±0.50	0.519±0.30	1.20±0.25	18.32	28.12
F8	0.419±0.30	0.515±0.28	1.28±0.23	19.08	29.17
F9	0.411±0.28	0.509±0.29	1.24±0.22	18.44	28.17

Table 4. Evaluation Parameters of Etodolac ODTs

Formulation Code	Weight variation[a](mg)	Thickness[b] (mm)	Hardness[b] (kg/cm^2)	Friability[c] (%)	Drug content (%)
F1	298.05±0.62	3.10±0.10	2.92±0.30	0.59	85.48±0.45
F2	299.01±0.51	3.05±0.12	2.78±0.23	0.65	89.01±0.49
F3	297.03±0.61	3.15±0.09	2.95±0.21	0.58	92.27±0.23
F4	298.01±0.67	3.12±0.11	2.83±0.46	0.68	93.60±0.40
F5	291.12±0.39	3.13±0.16	2.85±0.42	0.41	95.70±0.30
F6	293.05±0.52	3.14±0.14	2.96±0.56	0.47	98.01±0.09
F7	295.25±0.45	3.05±0.10	2.93±0.36	0.56	94.05±0.45
F8	299.01±0.28	3.13±0.05	2.82±0.40	0.45	98.07±0.13
F9	300.01±0.09	3.12±0.07	2.98±0.39	0.58	99.09±0.11

a. Mean ± S.D., n = 20, b. Mean ± S.D., n = 6, c. Mean ± S.D., n = 10 tablets

Table 5. Formulation characteristics of Etodolac ODTs

Formulation code	Disintegrating time[a] (sec)	Wetting time[b] (sec)	Water absorption ratio[b] (%)	*In vitro* dispersion time[c] (sec)
F1	32.41±0.24	25.02±0.48	30.56±0.45	38.94±0.25
F2	29.94±0.56	19.23±0.33	24.66±0.50	34.22±0.55
F3	28.35±0.18	17.35±0.50	22.52±0.29	31.87±0.21
F4	24.25±0.55	18.25±0.12	20.85±0.28	30.51±0.78
F5	20.25±0.28	15.25±0.84	18.28±0.84	27.84±0.70
F6	18.47±0.65	12.47±0.25	16.18±0.22	25.54±0.80
F7	22.04±0.51	11.04±0.18	15.58±0.55	22.35±0.57
F8	17.48±0.22	10.48±0.55	14.32±0.88	21.14±0.74
F9	15.05±0.65	08.39±0.22	12.77±0.55	18.56±0.25

a. Mean ± S.D., n = 6, b. Mean ± S.D., n = 10, c. Mean ± S.D., n = 3

Conclusion

The anti-inflammatory effects of Etodolac result from inhibition of the enzyme cyclooxygenase (COX). This decreases the synthesis of prostaglandins involved in mediating inflammation.

Etodolac oral disintegrating tablets were prepared by direct compression method using Crospovidone, Croscarmellose sodium, and sodium starch glycolate as super disintegrants exhibited good preformulation properties.

Based on disintegration and dissolution results, formulation F9 was the best formulation with crospovidone from prepared ODT.

Oral disintegrating tablets of Etodolac were found to improve the versatility, convenience, and

Table 6. Cumulative % Etodolac released from ODTs containing varying concentrations of different Superdisintegrating agents individually

Formulation code	Time (minutes)					
	5	10	15	30	45	60
F1	28.26±0.18	36.31±0.19	56.36±0.10	67.68±0.32	75.27±0.27	85.40±0.12
F2	35.64±0.20	41.22±0.10	58.58±0.17	69.48±0.11	80.36±0.28	89.09±0.21
F3	35.87±0.17	54.12±0.31	65.37±0.11	78.48±0.39	82.48±0.09	92.27±0.13
F4	30.06±0.51	40.44±0.15	66.96±0.05	76.56±0.09	87.48±0.51	93.06±0.39
F5	28.44±0.09	49.81±0.30	64.08±0.07	82.2±0.27	93.78±0.34	93.05±0.37
F6	30.42±0.08	52.02±0.09	74.16±0.12	89.65±0.35	92.25±0.46	98.01±0.09
F7	21.18±0.43	48.52±0.12	57.78±0.27	68.22±0.13	83.73±0.22	94.05±0.15
F8	35.59±0.17	52.02±0.18	68.85±0.38	74.56±0.24	84.77±0.19	98.07±0.23
F9	38.07±0.13	52.06±0.30	71.05±0.31	76.25±0.18	88.65±0.01	99.09±0.11

Mean ± S.D., n = 3

Table 7. FTIR Studies of Etodolac pure drug and optimized formulation

PEAK OF FUNCTIONAL GROUPS {WAVELENGTH (cm^1)}				
IR SPECTRA	N-H Stretching	C-O Stretching	C-N Stretching	C-H Stretching
Etodolac	3350	1397	889	2966 (sp^3)
Optimized formulation (F9)	3351	1422	1042	2915

patient compliance leading to an enhanced approach for the administration of the drug.

The orodispersible tablets are particularly beneficial to the pediatric, geriatric, bedridden, and psychotic patients affected by dysphagia. These tablets get converted into a suspension with the salivary fluid in the oral cavity thereby showing rapid onset of action with improved bioavailability, better patient acceptance and offer better safety as compared to conventional oral dosage forms.

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Optimization of *in vitro* conditions for induction of somatic embryos and regeneration of plantlets in *Euphorbia hirta* L.

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Abstract

Plantlet regeneration methods through somatic embryogenesis were standardized from stem explants of *Euphorbia hirta* in this report. The explants were surface sterilized with 0.1% (w/v) mercuric chloride solution. The sterilized explants were inoculated on MS (Murashige and Skoog) medium to induce somatic embryos. The explants with somatic embryos were transferred to the embryo germination and shoots elongation medium. The explants responded maximum on MS medium augmented with 0.4 mg/L 6-benzyleaminopurine (BAP) and kinetin (Kin) combined with 0.5 mg/L á-naphthalene acetic acid (NAA). About 93% response was observed when indole-3 acetic acid (IAA) was used at the place of NAA in the medium with BAP and Kin. Sucrose at 3% concentration was reported optimum for the induction of somatic embryos. Somatic embryos were differentiated into plantlets within 4 weeks on half strength MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L Kin. Maximum 83.9±0.51 plantlets (average length of 5.3±1.60 cm) per culture vessel were yielded on this medium combination after 4-5 subcultures. The induction of somatic embryos from the epidermal and subepidermal cells of the explants was confirmed by the microscopic observations of the transverse sections of explants with the embryoids. The methods were developed and the conditions were optimized for the induction of somatic embryos from the nodal explants of *E. hirta* in this study.

Keywords: *Euphorbia hirta*; somatic embryogenesis; MS medium; microscopic observations.

Introduction

Euphorbia hirta L. (family Euphorbiaceae) is an important medicinal herb, belongs to the tropical parts of the India, Bangladesh, Africa, America and Australia. The plant is popularly known as Pill bearing spurge and Basri dudhi (Hindi). It is a slender, hairy plant with profuse branches, which can spread up to the height of 40 cm (1). The plant is well acknowledged for its various medicinal properties. Traditionally, it is used for the treatment of asthma, bronchitis, ulcer, amoebic dysentery, diarrhea and dengue fever (2). The latex from the plant is used in treating hypertension, anemia, skin diseases, jaundice, edema, warts, sprains, inflammation, epilepsy and malaria (3).

Euphorbia hirta is explored for its valuable pharmacological activities like antibacterial, antifungal, antimalarial, anthelmintic, anticatarrhal, antidiarrheal, diuretic, antihypertensive, anxiolytic, anti-inflammatory, antidiabetic, antispasmodic, anticancer, antioxidant, antiasthmatic and antisyphilitic activities (2,3).

The various bioactivities of *E. hirta* are influenced by the presence of secondary metabolites such as flavonoids, polyphenols, tannins, sterols, alkaloids, glycosides and triterpenoids (4). The whole plant is reported to possess afzelin, myricitrin, rutin, quercitin,

euphorbin, gallic acid, protocatechuic acid, â-amyrin, â-sitosterol, heptacosane, nonacosane, shikmic acid, tinyatoxin, choline, camphol, rhamnose etc. The latex is endowed with inositol, taraxerol, friedelin, ellagic acid and kaempferol (5-7).

In vitro induction of somatic embryos is a preferred method of crop improvement programs (8). It provides a model system to explore the genetic basis of early differentiation events and cellular totipotency of somatic cells (9,10). The direct somatic embryogenesis has been reported in many plants like, *Prunus persica* (11), *Kalanchoe blossfeldiana* (12), *Nicotiana tobacum* (13), *Begonia pavonina* (14) etc. Somatic embryogenesis reduces the time required for complete plant regeneration which is advantageous in reduction of culture-induced genetic changes (15,16). Somatic embryogenesis has also been demonstrated in *Leptadenia pyrotechnica* (17), *Wedelia calendulacea* (18) etc. for the conservation of endangered germplasms.

The large scale production of plantlets through somatic embryogenesis would offer a stable alternative to meet the pharmaceutical demand of this plant. Barring few reports on the *in vitro* propagation of *E. hirta* to date (19), there is no report on direct regeneration through somatic embryogenesis. Therefore, the aim of the present study was to develop an efficient regeneration protocol through somatic embryogenesis and optimization of *in vitro* conditions for the induction of somatic embryos in *E. hirta*.

Materials and Methods

Explant material and surface sterilization : Different types of explants (node, internode and leaves) of *E. hirta* were procured from a three months old young emerging plant growing in the institute campus. The explants were isolated and cleaned under running tap water for 10-15 min, treated with 3% (v/v) Sodium hypochlorite (disinfectant) for 5-8 min. and rinsed five times with sterilized distilled water. The explants were subjected to a broad spectrum systemic fungicide 0.1% (w/v) Bavistin (BASF India Ltd.) for 5-6 min. Surface sterilization was carried out with 0.1% (w/v) mercuric chloride solution for 3-5 min and rinsed with sterile distilled water to remove the sterilant under aseptic conditions.

Culture medium and culture conditions : The surface sterilized explants were cultured on Murashige and Skoog (MS) medium (20) containing 0.8% agar and additives (50 mg/L ascorbic acid, 25 mg/L each of adenine sulphate, L-arginine and citric acid) with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 0.2-1.0 mg/L), 6-benzylaminopurine (BAP; 0.2-1.0 mg/L), kinetin (Kin; 0.2-1.0 mg/L), indole 3-acetic acid (IAA; 0.2-1.0 mg/L) and á-naphthalene acetic acid (NAA; 0.2-1.0 mg/L) (Himedia, Mumbai, India) for the induction of somatic embryos. Hormone free MS medium served as control. The pH of the medium was adjusted to 5.8 ± 0.02 with NaOH and / HCl prior to autoclaving. The media was dispensed into culture vessels and autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 15 min. All the cultures were maintained at 25±2°C with 16:8 (light:dark) photoperiod at 40–50 ìmol m^{-2} s^{-1} spectral flux photon density (SFPD) under cool white fluorescent tubes (Philips, Mumbai, India).

Induction and maturation of somatic embryos : The explants were cultured on different strengths of MS media augmented with different concentrations and combinations of growth regulators for the induction of somatic embryos. The explants were inoculated horizontally and vertically on growth medium and incubated at above mentioned *in vitro* conditions. After the induction of embryogenic masses on the surface of the explants, these were transferred to MS basal medium supplemented with growth regulators for the development and maturation of somatic embryos. The early stage somatic embryos were transferred to MS medium augmented with or without BAP and different concentrations of sucrose (1–5 % w/v) for the maturation of somatic embryos. After four weeks, the explants with somatic embryos were transferred to the multiplication medium and maintained under dark conditions at room

temperature for further multiplication of somatic embryos.

Germination of somatic embryos and elongation of shoots : The meristemoid portion with mother explants were excised and cultured on different strengths (full, ½ and 1/4th) of MS medium supplemented with different concentrations of BAP and Kin (0.25, 0.5 0.75 and 1.0 mg/L) for the germination of somatic embryos. Initially cultures were maintained in dark for a week. Once the embryogenic potential gained bythe explants on MS medium, the proembryo masses were transferred to fresh medium for further elongation of the shoots.

Photomicrography of somatic embryos : The somatic embryogenesis was further confirmed through microscopic observations. Fresh embryogenic tissues containing somatic embryos at different developmental stages were withdrawn from the cultures and fixed primarily in FAA solution (formalin: acetic acid: ethyl alcohol at 1:1:3 ratio) for 48 hrs and stored in 70% ethanol at room temperature. Free hand sections were done using a sharp razor. Sections were stained with 1% aqueous safranin (w/v) and mounted onto the glass slides. Photomicrographs of different magnifications were taken under bright light field using Olympus Stereomicroscope (SZ61) and Labomed iVu photomicroscope. The images captured were analyzed using Pixelpro software.

Acclimatization of plantlets : The well developed plantlets regenerated from somatic embryos were transferred to paper cups containing autoclaved soilrite® (Keltech Energy Limited, Bangalore, India). The plantlets were covered with a polyethylene bags with minute holes to maintain high humidity and irrigated weekly with tap water. After 4 weeks, the plantlets were shifted to nursery polybags filled with the mixture of red soil, garden soil and vermi-compost (1:1:1) for hardening. Finally, the plants were transferred to the field after two months.

Experimental design and statistical analysis: All the experiments were conducted with 20 replicates per treatment and the experiments were repeated thrice. The percentage of embryo induction and germination from stem explants was calculated. Data were recorded after 3 weeks in each experiment. The results were expressed as mean ± SE of three independent experiments. The data were analyzed statistically using one-way ANOVA and the significance in variation between responses with reference to various concentrations of hormones were assessed by Duncan's multiple range tests at a 5% probability level by SPSS version 16 (Chicago, USA).

Results

Induction and maturation of somatic embryos: Among the three types of explants used, maximum percentage of response in induction of somatic embryos was observed with inter – nodal explants than with the node and the leaf explants. The development of somatic embryos directly on the surface of explants was clear within a week of inoculation on MS medium (Fig. 1A). The surface of the explants is fully covered by somatic embryos of different stages within four weeks.

The somatic embryos formation was not recorded with the medium containing 2,4-D. Of the growth regulators used to induce somatic embryos, combination of cytokinins and auxins in the medium elicited better response. Somatic embryos were induced on all concentrations of BAP and Kin augmented with IAA or NAA. Application of NAA produced higher percentage of somatic embryos than IAA. MS medium supplemented with 0.4 mg/L each of BAP and Kin and 0.5 mg/L NAA resulted in good response of somatic embryo induction. Cent percentage response was observed on this medium combination. Maximum 92.9% induction was resulted when IAA was used at the place of NAA in the medium. Less percentage of somatic embryos were induced on BAP and Kin when incorporated alone in the medium (Table 1).

Full strength MS medium with 3% sucrose was reported optimum for the induction of somatic embryos in *E. hirta*. Lower and higher concentrations than 3% sucrose was not found

impressive in the induction frequency of somatic embryos in this study (Table 2).

Germination of somatic embryos and elongation of shoots : The germination of somatic embryos into plantlets was achieved by subculturing of mature and premature embryos to half strength MS medium containing low concentrations of cytokinins (BAP and Kin). Somatic embryos were developed into plantlets with the normal developmental stages to root and shoot differentiation within 4 weeks, when the meristemoids transferred onto half strength MS medium + additives and fortified with 0.5 mg/L BAP and 0.1 mg/L Kin (Fig. 1B).

The strength of MS medium significantly affected the regeneration of shoots from the somatic embryos. The embryos failed to germinate on full strength MS medium, even one-fourth strength of MS medium was also reported comparatively less effective in the germination of somatic embryos. The regeneration of the somatic embryos was clearly visible and green tiny shoots primordia were observed within week of transfer to the fresh medium. The green shoots emerged out from the green mass of cells after another one week (Fig. 1C-D). The regular subculturing of somatic embryos on fresh medium with the same composition and combinations yielded 83.9±0.51 plantlets per culture vessel (size 400 ml) with an average length of 5.3±1.60 cm (Fig. 1E-F and Table 3). The number of plantlets germination from the embryos was decreased gradually beyond the optimum concentrations of BAP and Kin.

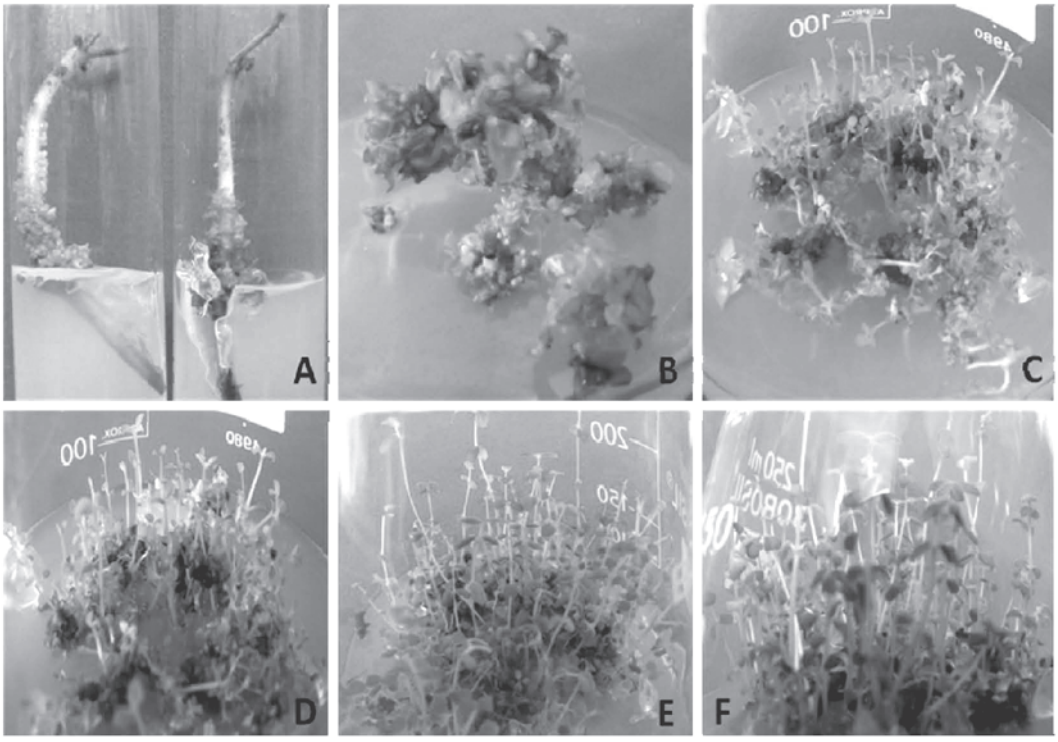

Fig. 1A. Somatic embryo on the surface of the explants. **Fig. 1B.** Germination of somatic embryos. **Fig. 1C.** Tiny green plantlets emerged out from the somatic embryos. **Fig. 1D.** Small shoots on half strength MS medium. **Fig. 1E.** Micropropagation of shoots on MS medium. **Fig. 1F.** Elongated shoots of *E.hirta*.

Maximum 15.4±0.23 shoots were germinated on hormone free MS medium.

Microscopic analysis of somatic embryos : The microscopic analysis of the cultures confirmed the induction of somatic embryos from the surface of the explants on full strength MS medium containing BAP, Kin and NAA/IAA (Fig. 2A). The clusters of green mass on explants surface showed pre-globular pro-embryos after 7 days of inoculation (Fig. 2B-C. The microscopic sections of explants tissue showed that the formation of somatic embryos occurred from the epidermal and subepidermal cells (Fig. 3A). Incubation under dark conditions promoted the development of globular somatic embryos. The globular embryos were less as compared to the bifurcated heart-shaped, and torpedo stages of embryos in the second weekof cultures (Fig. 3B). The different types of morphogenic responses were observed with time duration of the cultures. Globular and heart-shaped embryos were prominent in the first two weeks (Fig. 3C). Later at third and fourth weeks, torpedo and cotyledonary stages were prominent. The different stages of somatic embryos were detected on the same explants (Fig. 3D). Finally the mature embryos were developed into the shoots and roots.

Discussion

Somatic embryogenesis opens a promising opportunity to regenerate large number of identical

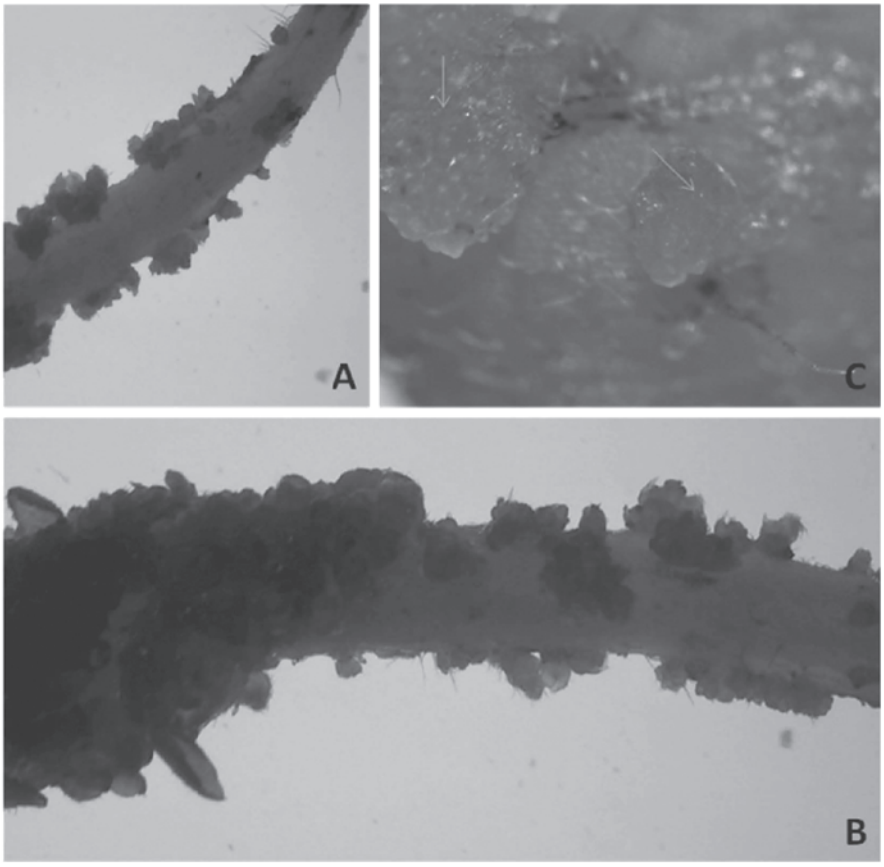

Fig. 2A. Microscopic image of the somatic embryos on the surface of the explant.
Fig. 2B. Early stages in the development of somatic embryos in *E. hirta*.
Fig. 2C. Different stages in the development of somatic embryos with the explants.

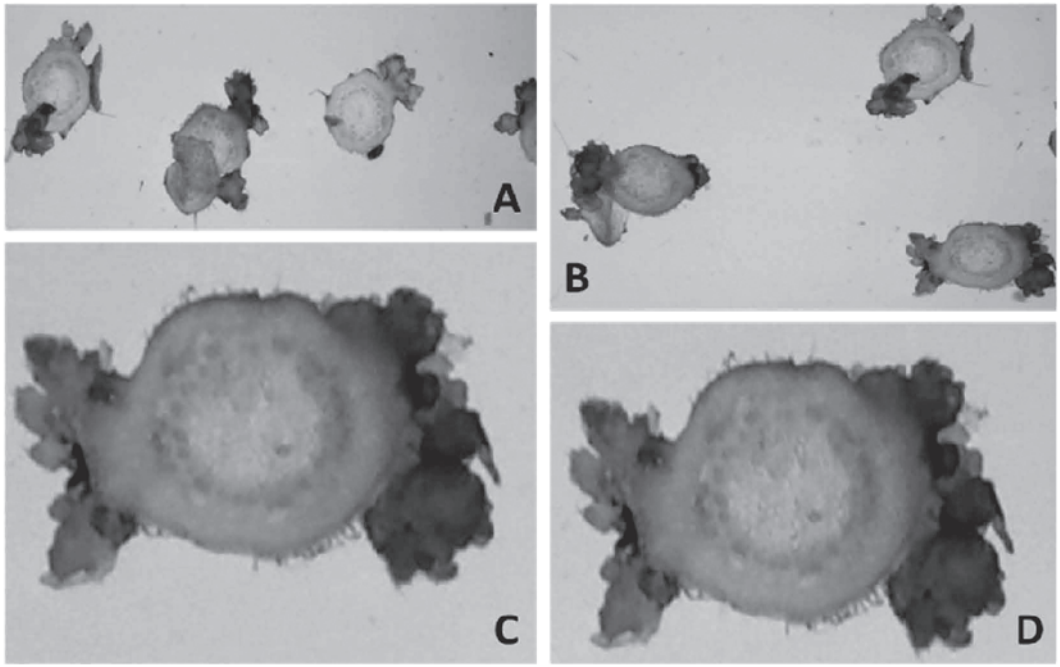

Fig. 3A. Transverse sections of the somatic embryos with the explants.
Fig. 3B. Bifurcated heart-shaped stages of embryos after two weeks of incubation.
Fig. 3C. Globular and heart-shaped embryos.
Fig. 3D. Different stages of somatic embryos after four weeks of incubation.

plantlets within short duration of time (13). Maximum percentage of response in induction of somatic embryos was observed with inter-nodal explants in this study. The response of explants on *in vitro* condition is genotype independent (21). Somatic embryos were induced from several of explants in number of Euphorbiaceae members (19). Marconi and Radice (22) reported somatic embryogenesis using leaf explants in *Codiaeum variegatum*. Immature anthers were used as explants in *Hevea brasiliensis* (23), mesophyll cells in *Euphorbia nivulia* (24), nodal segments in *Euphorbia pulcherrima* (25) and leaf and floral tissues in *Manihot esculenta* (26).

The embryos formation was not recorded with the MS medium augmented with 2,4-D but the combination of cytokinins and auxins in the medium elicited better response in this study. Martin et al. (24) induced maximum percentage of somatic embryos in E. nivulia using BAP and 2,4-D in contrast to these results. Singh *et al.* (27) reported that the presence of 2,4-D in the medium produced negligible amount of somatic embryos than the presence of BAP in *Sapindus mukorossi*. The addition of cytokinin with 2,4-D could enhance formation of the somatic embryogenesis than the 2,4-D alone (28).

The MS medium supplemented with 0.4 mg/L each of BAP and Kin and 0.5 mg/L NAA resulted in good response of somatic embryo induction. The combination of Kin and NAA induced maximum number of somatic embryos in *H. brasiliensis* (23). Somatic embryogenesis have been reported in several Euphorbiaceae members such as *M. esculenta* (29), *E. pulcherrima* (30), *E. nivulia* (24), *Jatropha curcas* (21) etc. The influence of growth regulators and culture environments were reported to be crucial in the development of somatic embryos in number of

Table 1: Effect of auxins and cytokinins on induction of somatic embryos in *E. hirta*.

BAP(mg/L)	Kin(mg/L)	IAA (mg/L)	NAA (mg/L)	Explants showing somatic embryogenesis (%)
0	0	0	0	0^{a}
0.2	-	-	-	83.9^{ij}
0.4	-	-	-	91.4^{n}
0.6	-	-	-	88.0^{m}
0.8	-	-	-	81.9^{h}
1.0	-	-	-	76.2^{f}
-	0.2	-	-	56.0^{b}
-	0.4	-	-	72.3^{e}
-	0.6	-	-	69.7^{d}
-	0.8	-	-	63.1^{c}
-	1.0	-	-	57.5^{b}
0.2	0.4	0.5	-	83.0^{hi}
0.4	0.4	0.5	-	92.9^{m}
0.6	0.4	0.5	-	89.6^{m}
0.8	0.4	0.5	-	85.0^{jk}
1.0	0.4	0.5	-	77.5^{fg}
0.2	0.4	-	0.5	86.3^{kl}
0.4	0.4	-	0.5	100^{o}
0.6	0.4	-	0.5	91.8^{n}
0.8	0.4	-	0.5	87.7^{l}
1.0	0.4	-	0.5	79.2^{g}
LSD				3.42

Note: Mean values followed by same alphabet not differing significantly at 5% level according to Duncan's Multiple Range Test.

Table 2: Effect of different concentrations of sucrose on induction of somatic embryogenesis.

Sucrose concentration (%)	Induction of somatic embryos (%)
1.0	43.0^{a}
2.0	51.5^{b}
3.0	93.9^{e}
4.0	70.6^{d}
5.0	66.4^{c}
LSD	2.01

Note: Mean values followed by same alphabet not differing significantly at 5% level according to Duncan's Multiple Range Test.

Table 3: Effect of BAP and Kin on germination of somatic embryos on half strength MS medium after 4 weeks.

Conc. of BAP (mg/L)	Conc. of Kin (mg/L)	No. of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)
0	0	15.4±0.23^{a}	1.2±0.16^{a}
0.5	0.1	83.9±0.51^{b}	5.3±1.60^{e}
1.0	0.1	79.0±0.57^{h}	4.7±0.91^{de}
1.5	0.1	74.1±0.05^{g}	4.4±1.00^{d}
2.0	0.1	65.9±1.15^{f}	3.7±0.63^{c}
2.5	0.1	60.3±0.17^{e}	3.0±0.17^{b}
3.0	0.1	55.8±0.28^{d}	2.9±0.59^{b}
LSD	3.91	1.24	

Note: Mean values followed by same alphabet not differing significantly at 5% level according to Duncan's Multiple Range Test.

Euphorbiaceae plant species. Linossier *et al.* (31) studied the effect of ABA and high concentration of polyethylene glycol on induction of somatic embryos in *H. brasiliensis*.

Full strength MS medium with 3% sucrose was reported optimum for the induction of somatic embryos in this investigation. Rambabu *et al.* (32) induced zygotic embryos in *Givotia rottleriformis* with the same concentrations of sucrose but Konnan *et al.* (33) initiated somatic embryos with 2% sucrose. The present results are similar with the somatic embryogenesis in Rose plant, *Curculigo orchioides* and *S. mukorossi* (33, 34, 27), where maximum percentage of somatic embryos was induced with 3% sucrose in the medium.

The embryos were unable to germinate on full strength MS medium, even one-fourth strength of MS medium was also reported less effective in E.hirta. Groll *et al.* (29) suggested half and full strength MS salt strength more suitable in somatic embryogenesis of *M. esculenta*. Cheruvathur *et al.* (34) reported half strength MS medium optimum for the germination of somatic embryos in *Rhinacanthus nasutus*. Germination of somatic embryos was confirmed by the development of bipolar structures leads to the formation of shoot and root from the mass of tissues. Similar observations were recorded in *Decalepis hamiltonii* (37), *Tylophora indica* (38) and *R. nasutus* (36).

The regular subculture of somatic embryos on fresh medium with BAP yielded maximum plantlets. The essentiality of BAP in the medium for germination of somatic embryos was also reported in *Phoenix dactylifera* (39), *W. calendulacea* (18) and *S. mukorossi* (27). Combination of BAP and NAA was found optimum for morphogenesis of somatic embryos in *E. nivulia* (24).

The microscopic examinations confirmed the origin of somatic embryos from the surface of the explants. The formation of direct somatic embryos from surface of the explants was also reported in *Arabidopsis thaliana* (10). Rai *et al.* (40) observed that the induction of somatic embryos is determined by the specific tissues of explants during developmental stages. The stages of somatic embryos from meristematic region to step wise globular, heart and torpedo stages through microsocopical analysis were also reported in *P. dactylifera* (39) and *Nicotiana* sp. (13). The morphogenesis of somatic embryos and plantlet conversion has been studied in *L. pyrotechnica* (17), *Elaeis guineensis* (41) and *Hybanthus enneaspermus* (42).

Conclusion

The somatic embryos could be directly produced from the surface of the explants of *E.hirta* These can be elongated and developed into shoot and root on different strengths of MS media. The embryos were originated from the epidermal and subepidermal cells of the explants as confirmed by the microscopic observations of the sections of explants. The present investigation could be explored for the large scale plant production and germplasm conservation of multipotent medicinal plant *E. hirta*.

Conflict of interests : Authors declare that there is no conflict of interests in this publication.

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In Vitro Fermentation of *Acacia Senegal* by Fecal Microbiota from lean Donors to Stimulate the Growth of Probiotic

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Abstract:

Gum Arabic is believed to have many health benefits including prevention of gastrointestinal diseases. Prebiotic resist digestion in the upper gastrointestinal tract and allowed for stimulation of bacterial growth in the distal intestine and colon. The prebiotic properties of *Acacia Senegal* was studied using mixed cultures of human fecal bacteria from four lean individuals. The results obtained were compared with inulin as positive prebiotic control. Fermentation studies were carried out using anaerobic, pH-controlled faecal batch cultures, and the changes in the faecal microbial population were monitored at 0, 6, 12, 24 and 36h by fluorescent in situ hybridization (FISH). Quantitative FISH results revealed that *Bifidobacterium*spp. *Bacteroidacea* spp. and *Lactobacillus* spp. were selectively increased ($P<0.05$) after the fermentation of *Acacia Senegal* by the fecal microbiota.*Clostridium* spp.,even have showed slight increase in fermentation of *Acacia Senegal*and inulin but was not significant. The stimulation of growth of probiotic bacteria was accompanied by a high production of acetate acid. The fermentation of *Acacia Senegal* may help to improve health through stimulation of bacteria growth which led to production of acetate. This study provides proof for the prebiotic effectiveness of *Acacia Senegal*, and the result showed that it might have a prolongedbifidogenic effect, thus could prevent certain types of diseases.

Keywords: prebiotic; *Acacia Senegal*; Colonic fermentation; probiotic; SCFA

Introduction:

Gum Arabic, known as acacia gum is a natural gum made of exudation taken from two species of the acacia tree; *Senegalia senegal* and *Vachellia seyal*. The gum is harvested commercially from wild trees throughout Sudan. It is a complex mixture and heteropolysaccharide of high molecular weight of glycoproteins and polysaccharides. It was historically the source of arabinose sugars, which was first discovered and isolated from it (Calame, *et al*.,2008).*Acacia gum*, is traditionally utilized by African populations (Cherbut*et al.* 2003) to prevent and treat intestinal disorders. There has been illustrated that gum feeding can improve intestinal transit and provide digestive comfort (Cherbut*et al.* 2003). Such traditional belief in promoting characteristics of prebiotic have been used and accepted. A prebiotic is a "nondigestible food ingredient that affects the host by selectively targeting the growth and/or activity of one or a limited number of bacteria in the colon, and thus has the potential to improve host health".(Hughes *et al.* 2007). Prebiotic has been shown to improve health and prevent diseases, with the more soluble, fermentable prebiotic sources being proposed to reduce the glycaemic index, insulin sensitivity (Hallfrisch &

Behall 2000) and cholesterol absorption (Kahlon*et al*. 1994) by the simulation of gut microbiota.

The gastrointestinal tract is a complex ecosystem containing up to 10^{11} CFU of bacteria per gram of intestinal content. This large population of bacteria plays a key role in normal gut function, human health and well-being (Van, *et al* 2004). In particular, *Bifidobacteria* and *lactobacilli* are believed to play major important role in maintaining intestinal health and are generally regarded as probiotics due to their effects on maturation and balancing of the immune system (Peran, L. *et al*. 2007; Sartor, R. B. 2008;Zeuthen, L. H.*et al.* 2010) and to their ability in inhibition of pathogens (Collado. M.C.*et al.*2007., Fukuda, S. *et al.* 2011.). However, a sufficient prebiotic intake is required for the desired effect (European Food Safety Authority. 2010). The selective stimulation of specific colonic bacteria is explained by the capability of these bacteria to break down the glycosidic linkages in the prebiotic carbohydrates. These bacteria are able to grow on particular carbon sources, which are less easily fermented by other members of the intestinal community. This provides those bacteria with a selective advantage when competing with other bacterial species in a mixed bacterial community such as the human colon (Sanz, M. L., *et al*. 2005). Hence, there is a great interest in the manipulation of the intestinal microbiota, targeting to increase of the number of *bifidobacteria* and *lactobacilli* and to stimulate the production of short chain fatty acids (SCFA) and lactate in the colon (Gibson G.R & Roberfroid M.B. 1995; Pool-Zobel B.L. 2005). Therefore, this study may provide information regarding prebiotic properties within the *Acacia Senegal*. A small-scale faecal batch culture representing the human gut was used in the experiment. The fermentability and bifidogenic activity of different *Acacia Senegal* was monitored by bacteria enumeration using FISH method. Short-chain fatty acid (SCFA) production was also determined by high performance liquid chromatography (HPLC).

Material and Methods:

Samples*:* The Gum Arabic (*Acacia Senegal*) sample used in this study was purchased from a Sudanese market.Inulin from chicory root (Warcoing, Belgium), being an established prebiotic ingredient, was used as a positive control.

Faecal inoculation : Fecal samples were provided by four healthy adult donors (male, aged 20–30 years old) and (BMI: 18.5-24.9 kg/m2). The donors, had not received antibiotic treatment and had not consumed pre or probiotic supplements for at least six months before the study. Freshly stool samples were collected in sterile plastic pots at The University of Putra Malaysia, Bintulu campus on the day of inoculation of the batch vessels. Samples were diluted (1:10 w/w) with sterile phosphate buffered saline (PBS) (0.1 M, pH 7.4) and homogenized in a stomacher (Stomacher 400, Seward,West Sussex, UK) at normal speed for 2 min.

In vitro fermentations: Anaerobic, pH controlled batch culture fermentations with customised glassvessels (Soham Scientific, Fordham, UK) were used to assess the effect of *Acacia Senegal* on composition of gut microbiota and fermentation characteristics. Fermenter vessels (100 ml) with only 50 ml working volume were aseptically filled with 45 ml of presterilized basal nutrient medium (Sarbini*et al*. 2011). The medium contained the following ingredients: (2 g/l peptone water (Oxoid), 2 g/l yeast extract (Oxoid), 0·1 g/l NaCl, 0·04 g/l K2HPO4, 0·04 g/l KH2PO4, 0·01 g/l MgSO4.7H2O, 0·01 CaCl2.6H2O, 2 g/l NaHCO3, 2 ml Tween 80 (BDH), 0·05 g/l haemin, 10µl vitamin K1, 0·5 g/l cysteine.HCl, and 0·5 g/l bile salts, pH 7·0) and continuously sparged with O_2 free nitrogen (15 ml/min) and each vessel was magnetically stirred. The test sample (*Acacia Senegal*) (1% w/w) were added at 0.5g in 50ml working volume, just before addition of 10% fresh fecal slurry. Control fermentations supplemented with inulin as positive prebiotic control at a concentration of 1% w/w were also included. The temperature of each batch vessel was maintained at 37 C by means of a circulating water bath. The pH was maintained at 6.8 using an automated pH

controller (Fermac 260, Electrolab, Gloucestershire, UK). Batch cultures were run for a period of 36 h and 5 ml samples were obtained from each vessel at 0, 6, 12, 24 and 24 h for FISH and SCFA analysis. The batch experiments were performed in four replicates with four different fecal donors for each substrate.

Bacterial enumeration by Fluorescent in situ hybridization (FISH): Bacterial composition in the gut models was analyzed using Fluorescence *in situ* hybridization (FISH). Synthetic oligonucleotide probes bind with specific regions of the 16 S ribosomal ribonucleic acid molecule and labelled with the fluorescent dye Cy3 were utilised for the enumeration of bacterial groups (Table 1). A volume of 375 µl of samples from each vessel at each sampling time were fixed for 4h at 4 C° with 1125 µl of 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1:3 (v/v). Samples were washed twice with phosphate-buffered saline (0.1 M, pH 7.0) and centrifuged at 13 000 g for 5 min and washed twice in 1 ml filter-sterilized PBS. The Pellets were resuspended in 300 µL of filtered PBS/ethanol (99 %) in a ratio of 1:1 (v/v) and stored at -20 C° until further processing. Dilution steps were performed using 10 µL of the fixed samples added to suitable volume of PBS in order to obtain a countable number of fluorescent cells in each field of view. The bacteria were enumerated by adding 20µL to each well of a six-well polytetrafluoroethylene/ poly-L-lysine-coated slide (Tekdon Inc., Myakka City, FL). Slides were dried at 46C° for 15 min. After 15 min of drying, a dehydrated steps were applied, using ethanol with different concentration (50, 80 and 96%) each step of dehydrated take 3min. Slides were returned in the drying oven for 2min to evaporate excess ethanol before addition of hybridisation mixture. Hybridisations were performed by adding 50µLhybridisation mixture consisting of 45µLhybridisation buffer (5 M NaCl, 1 M Tris/HCl pH 8, 30% formamide, ddH2O, 10% SDS) and 5µL probe (Chis150, Lab158, Bac303and Bif164) to each well and left to hybridise for 4h in a microarray hybridisation incubator (Grant-Boekel, Cambridge, UK). Washing buffer was used direct after the hybridization of the slides for 15 min and then followed with dipping in cooled water for few seconds. Slides were dried using compressed air. Subsequently, 5 microliters of polyvinyl alcohol mounting medium with 1,4-diazabicyclo (2,2,2) octane were added onto each well and a cover slip was placed on each slide (20 mm; thickness no. 1; VWR, Lutterworth, UK). Numbers of total bacteria were determined by taking into account viewing each well for fifteen different fields using epifluorescence microscope (CX31; Olympus, Tokyo, Japan) using CX-RFL-2 reflected fluorescence attachment.(Sarbini*et al*. 2011).

Table 1. 16S rRNA oligonucleotide probes used in FISH analysis of bacterial populations.

Probe Code	Target group	Sequence (5'-3')	Reference
Chis150	Most of the Clostridium histolyticum group (Clostridium clusters I and II)	TTATGCGGTATTAATCTYCCTTT	Franks et al. (1998)
Lab158	Lactobacillus–Enterococcus	GGTATTAGCAYCTGTTTCCA	Harmsen et al. (1999)
Bac303	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	CCAATGTGGGGGACCTT	Manz et al. (1996)
Bif164	Bifidobacterium spp.	CATCCGGCATTACCACCC	Langendijk et al. (1995)

Short chain fatty acids analysis: The short chain fatty acids (SCFA) lactate, acetate, propionate and butyrate, were analysed by ion-exclusion HPLC system (SHIMADZU SPD-20A)equipped with UV-VIS detector. The column used was an ionexclusionRezex ROA-Organic Acid H^+ (8%), 300 X 7·80mm (Phenomenex). Sulphuric acid in HPLC-grade water (0.0025 mmol L^{-1}) was used as the eluent. Samples taken from the batch culture vessels were centrifuged at 13 000 g for 10 min to remove all particulate material. Supernatants were then filtered using 0.2 mm polycarbonate syringe filters (Millipore) and 15 µL was injected into an HPLC system. HPLC was operated at a flow rate of 0·5 ml/min with a heated column at 40^0C. The run time for sample was 40 min. Quantification of the samples was obtained by comparing with calibration curves of lactate, acetate, propionate, and butyrate in concentrations ranging between 6.25 and 100 mM.

Statistical Analysis : Statistical analysis of the data was performed by one-way ANOVA using (SPSS 23 software). Significant differences ($p<0.05$) among gum Arabic samples were analyzed by Duncan triplicates range test Bryman and Crame (2012).

Result:

Bacterial enumeration : Bacterial counts with the duration of 0h, 6h, 12h, 24h and 36h of incubation are shown in Table 2. After fermentation of *Acacia Senegal* and inulin in fecal slurries obtained from healthy donors, FISH method was applied to measure the concentration of selected bacterial. Fermentation of *Acacia Senegal* resulted in a significant increase in the content of *Bifidobacterium*spp.,*Bacteroidetes*spp., and *Lactobacillus* spp. *Acacia Senegal* showed no significant increase in the growth of *Bifidobacterium* from 0 until 12 h and also *Bacteroidetes*at 0h and 6 h, whereas, inulin showed significant growth of *Bifidobacterium* and *Bacteroidetes*from 0 to 24h. The time and speed of fermentation mostly effected by the structure of prebiotic which effect on the growth of probiotic.

Table 2. Mean value of bacterial population (log10 cells/ml batch culture fluid) in the colon model at 0,6, 12, 24 and 36 h inoculated with faecalmicrobiota (n = 4).

	Time	Bif164	Bac303	Lab158	Chis150
Senegal	0h	8.097±0.13	7.846±0.09	8.057±0.02	7.467±0.07
	6h	8.361^{b}±0.05	8.042^{b}±0.04	8.337*±0.06	7.571±0.18
	12h	8.376^{b}±0.1	8.146^{b}*±0.04	8.391*±0.05	7.585±0.07
	24h	8.656^{b}*±0.07	8.351^{b}*±0.05	8.454*±0.04	7.765±0.09
	36h	8.808^{a}*±0.09	8.677^{a}*±0.12	8.463*±0.08	7.612±0.06
Inulin	0h	8.097±0.13	7.846±0.09	8.057±0.02	7.467±0.07
	6h	8.577^{a}*±0.03	8.307^{a}*±0.05	8.308±0.08	7.696±0.09
	12h	8.856^{a}*±0.11	8.532^{a}*±0.07	8.545±0.11	7.687±0.19
	24h	8.938^{a}*±0.04	8.620^{a}*±0.07	8.561±0.12	7.588±0.23
	36h	8.830^{a}*±0.06	8.496^{a}*±0.08	8.489±0.22	7.693±0.26

Bif164: Bifidobacterium spp.; Bac303: Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae; Lab158: Lactobacillus/Enterecoccus; Chis150: Clostridium histolyticum. Mean value with unlike superscript letters (a,b) was significantly higher/lower in comparison between substrates in between the same sampling hour ($p < 0.05$).

*Mean value was significantly different from that of 0 h ($p < 0.05$).

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However, since *Acacia Senegal* is complex heteropolysaccharide with high molecular-weight so the fermentation might be slow and led to low growth of probiotics. The lower molecular-weight oligosaccharides such as (inulin) are rapidly fermented. This may be because the enzymes produced by Bifidobacterium spp. prefer to utilize non-reducing ends due to the low molecular mass. (Gibson. *et al*. 2004; Sarbini. S.R & Rastall R.A.2011).

Surprisingly, the growth of *Bifidobacterium*, *Bacteroidetes* and *Lactobacillus* start to drop for inulin at 36h (8.83, 8.49, 8.48 Log_{10}) respectively, while the growth of bacteria fermenting*Acacia Senegal* still dramatically increasing. However, the growth of *Bifidobacterium* and *Bacteroidetes* population at 36 h for *Acacia Senegal* showed no significant difference compared to fermentation of inulin. Our findings are in agreement with Wyatt *et al.* 1986, who noted an increase in the numbers of Bacteroides and Bifidobacterium. No significant difference in the growth of *Lactobacillus* population for inulin when compared to 0h.Both *Acacia Senegal* and inulin show no significant difference in the growth of *Clostridium* spppopulation when compared with 0h.However these results showed that, *Acacia Senegal* was selected by the probiotic bacteria.

Short-chain fatty acid (SCFA)analysis: Amounts of SCFA (lactate, acetate, propionate, butyrate) produced by fermentation of *Acacia Senegal* and Inulin is shown in Figure 1. Supplementation of *Acacia Senegal* to gut models inoculated with faecal samples from lean donors led to a significant increase of acetate at 24h and 36h as the major production of the fermentation(95.8 mM and 114.6 mM) respectively, whereas 6 and 12h show no significant growth when compared to 0h(53.06 mM and 46.05mM) respectively. Fermentation of inulin showed significant increase of acetate concentration at all times, but at 36 the production of acetate start to slowdown. Acetate produced may improves intestinal defence mediated by epithelial cells, which could protect the host against enteropathogenic infection (Fukuda. *et al.* 2011).

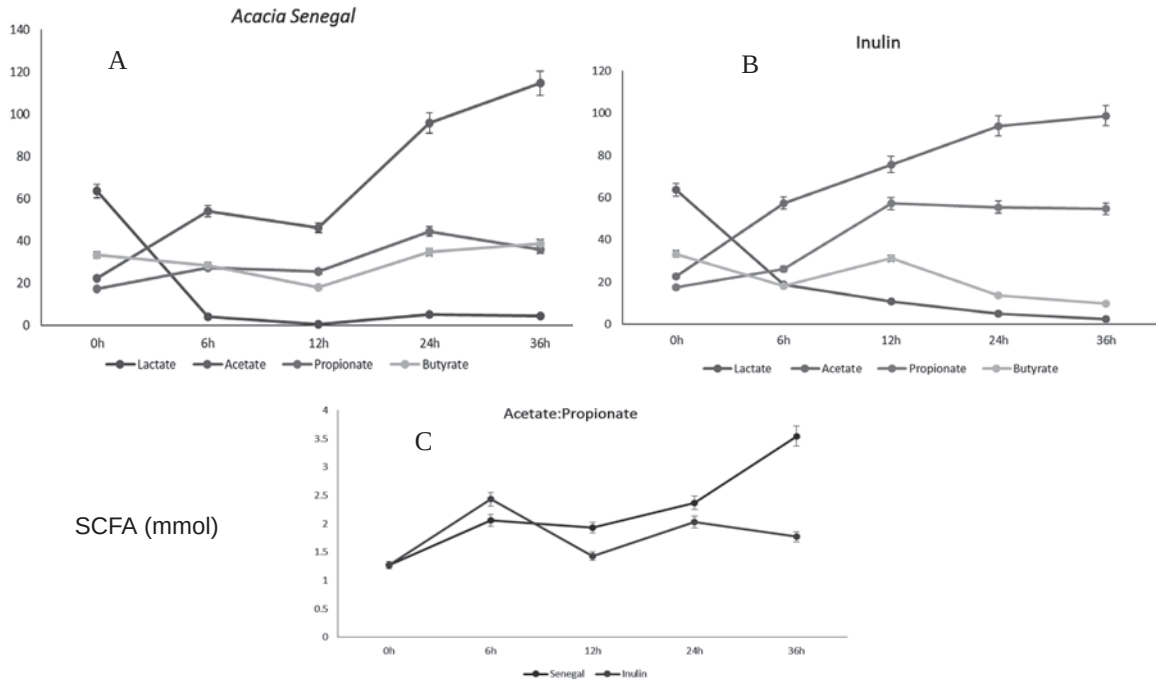

Fig 1: Cumulation production of SCFA (mmol)in pH-controlled batch cultures at 0,6,12 and 24 h (n = 4).

In Vitro Fermentation of *Acacia Senegal* by Fecal

No significant difference was observed in the amount of propionate and butyrate after fermentation of *Acacia Senegal.* Butyrate significantly decreased at *Acacia Senegal* fermentation at 6, 12 h but later elevate at 24, 36 h. No significant increase in butyrate was found in inulin. The butyrate produced may be due to the conversion from other SCFAs, i.e. acetate or lactate by cross-feeding.(Belenguer*et al.* 2006). The acetate to propionate ratio was dramatically increased ($P < 0.05$) by *Acacia Senegal*. Whereas, inulin slightly increased on the acetate to propionate ratio at 6h but later diminished at 12h. After that the ratio elevated with no significant different, this because of the changes on propionate concentration during the fermentation of inulin. The low acetate:propionate ratio may be of interest for regulating serum cholesterol concentrations, because acetate may act as a precursor for cholesterol synthesis, while propionate might inhibit this process (Delzenne. N.M &Kok N 2001; Wolever. *et al*.1995). Lactate production of *Acacia Senegal* and inulin were significantly decreased at all fermentation times from 0h to36h. This is because lactate may be further metabolised to acetate, propionate and butyrate by a number of cross-feeding bacteria.(Vernia. P *et al*. 1988; Sarbini*et al*. 2011).

Conclusion:

The results obtained from this study indicate that *Acacia Senegal* induced great modulation of *in vitro* faecal microbiota and comparable with inulin. *Bifidobacterium* spp and *Lactobacillus* ssp. growth was stimulated by *Acacia Senegal* throughout the fermentation resulting in high production of SCFA.

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Effect of Metal ions on the Dilute Acid Pretreatment of Lignocellulosic Biomass

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Abstract

Economically feasible, sustainable and non-polluting ways for ethanol production has become important due to limited petroleum supply and associated environmental consequences of burning fossil fuel. Three experimental lignocellulosic materials viz., *Hyptis suaveolens*, wheat straw and *Ailanthus exelsa* wood were taken to subject them to dilute acid pretreatment in presence of selected metals. Metals Zn and Ca were found general inhibitors of release of hemicellulosic sugars. *A. exelsa* wood was found to be more sensitive to metals in the release of hemicellulosic sugars during pretreatment by dilute acid method. Metals were also found to interfere with the utilization of sugar and ethanol fermentation by the yeast.

Key words: Pretreatment, metal ion effect, lignocelllulose.

Introduction

Fossils are supposed to have been produced by decomposition of dead plants and animals in millions of years, so fossil fuel is limited and non-renewable per se. Fossil fuels are organic hydrocarbons that on burning gives off carbon dioxide, a known greenhouse gas largely responsible for the climate change. In view of this, there is increased demand and interest in alternative fuel especially liquid transportation fuel all over the world (1). Transport fuel from biomass called biofuel thus emerged as the most important alternative fuel.

Of various biofuels, bioethanol as petroleum extender in the existing vehicles has been in use since 1970s and is expected to be used as singular fuel in future vehicles with suitably modified engine. Initial bioethanol called 1G bioethanol is obtained from starch crops such as corn and sugar cane. Since, IG ethanol has been blamed for its role in food verses fuel competition, attention has now been focused on 2G bioethanol whose feedstock is waste lignocellulosic materials (2).

Lignocellulosic biomass as feedstock is abundant and renewable and thus the product bioethanol is considered as the sustainable energy resources (3,4). Lignocellulose has fermentable glucose in polymerized form as cellulose which in turn is intertwined with lignin and hemicelluloses. In order to expose cellulose to enzyme or alternative physical method to release glucose from it, pretreatment of lignocellulosic is necessary. Of various pretreatment methods (5), dilute acid one of the most widely accepted method (6). The method applies 0.4-1% sulphuric acid to lignocellulosic material, the process often called impregnation followed by heat treatment (7). The reaction thus involves substrate lignocellulosics and three reagents viz., heat, acid and water. Of these three reagents the quality of former two is largely under control, the quality of natural supply of water however is extremely variable especially in terms of dissolved metals. At commercial level it is obvious that a very high quality of water may not be used everywhere and thus data about the role of various metal ions

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present in natural water in the efficiency of dilute acid pretreatment method must be available.

In the present study therefore the effect of some commonly occurring metals in natural water on the release of hemicellulose and lignin degradation products during dilute acid pretreatment from lignocellulosics was tested.

Materials and Methods

Plant materials : *Hyptis suaveolens* a wild weed found abundantly in north India, *Ailenthus exelsa* a potent energy plant (7,8) and a highly appreciated agro-waste viz., wheat straw were used as feed-stocks. The plant biomass of all the three materials was milled with particle size about 1mm x 0.5cm x 0.5cm, air dried to 10% moisture and stored at room condition until use.

Impregnation of biomass : Before the hydrolysis step, 100 gm of biomass from each source was impregnated with 0.7% v/v H_2SO_4 with 1mM of metal ions like Zn ($ZnCl_2$), Ca ($CaCl_2$), Fe ($FeCl_2$), Cu ($CuSO_4$) and Mg ($MgSO_4$) to a final volume of 1000 ml in separate flasks and incubated overnight. After the incubation, the excess fluid was removed. The treated woodchips were cooked at 121^{o}C for 30 min in autoclave. The wood prehydrolysate obtained by above process was recovered by filtration with Whatman No.4.

Treatment of hydrolysate :The hemicellulose acid prehydrolysate (HAP), was once more heated to 100^{o}C for 15 min to break down oligosaccharide into monomers of hexoses and pentoses. The hydrolysate was basified with solid NaOH (9) or over limed with limestone (10) until the pH reached 9.0 - 9.5. Insoluble residues from treated HAP were removed by filtration (Whatman No.4), and the supernatant was collected for further use as fermentable sugars (9). Triplicate samples of the hydrolysate were analyzed to estimate the amount of fermentable sugars.

Microorganism and its maintenance : *S. stipitis* (formerly *P. stipitis*) NCIM 3507 was obtained from National Collection of Industrial Microorganism (NCIM), NCL, Pune. It was maintained on agar slants containing (g l^{-1}): xylose, 20; yeast extract, 3; malt extract, 3; peptone, 5; and agar, 20. The medium used for inoculum preparation contained (g l^{-1}): D-xylose, 50; glucose, 5; yeast extract, 3; malt extract, 3; peptone 5; pH 5. To prepare the inoculum, a 250-ml Erlenmeyer flask containing 50 ml medium was inoculated with microbe from a fresh agar slant, and incubated at 30°C on a rotary shaker at 150 rpm for 48 h prior to use. A 5 ml aliquot of this culture was transferred to 100 ml fermentation medium.

Fermentation : Anaerobic batch fermentation of 100 ml broth consisting of 70% pretreated, detoxified hydrolysate of *H. suaveolens*, wheat straw and *A. excelsa* and 30% supplementary medium in separate conical flasks was carried out. Supplementary medium consisted of (g/l) glucose, 20; yeast extract, 3; peptone, 5; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0·5; trace element solution, 1 ml l^{-1}; the pH adjusted to 5·0 ± 0·1. The trace element solution contained (g l^{-1}): $CuSO_4.5H_2O$, 2·5; $FeCl_3.6H_2O$, 2·7; $MnSO_4.H_2O$, 1·69; $Na_2MoO_4.2H_2O$, 2·42; $ZnSO_4.7H_2O$, 2·87; $CoCl_2.6H_2O$, 2·38; and H_2SO_4 (conc.) 3 drops (11). Complete sterilization was done by autoclaving at 121^{o} C, 15psi for 30 min. Fermentation was carried out at temperature 32^{o}C with agitation at 100 rpm on shaker incubator for six days.

Analytical methods : Assay of total reducing sugar was carried out by DNS (dinitrosalycilate) method (12) pentose sugar by Orcinol reagent method (13). Ethanol was quantified in the distillate applying Potassium dichromate reagent method (14).

Statistical control : All experiments were performed in triplicates and three times. Statistical significance of the means was evaluated using one-way analysis of variance. Subsequent comparisons were performed using the least significant difference (LSD) test. Differences were considered significant when $P < 0.05$.

Results and Discussion:

Effect of metal ions on H. suaveolens wood pretreatment : As a result of pretreatment in presence of various metals, maximum negative impact was found with Zn that reduced release of

sugars to 30% level of that obtained without any metal. On the other hand least impact was found with Mg. All other metals showed negative impacts although their magnitude differed (Fig. 1). The release of phenolics was hardly influenced by the presence of tested metals.

Effect of metal ions on wheat straw pretreatment : In case of pretreatment of wheat straw in presence of various metals, maximum negative impact was found with Ca that reduced release of sugars to 20% level. On the other hand least impact was found with Mg. All other metals showed negative impacts although their magnitude differ (Fig. 2). In case of phenolics, the release was rather stimulated by Fe, Mg and Cu.

Effect of metal ions on A. exelsa wood pretreatment : In case of *A. exelsa* wood the result of pretreatment in presence of various metals showed maximum negative impact with Fe that reduced release of sugars to 20% level. On the other hand least impact was found with Mg. All other metals showed negative impacts with their magnitude varied between 70-80 % levels (Fig. 3). In case of phenolics, the release was influenced by the tested metals.

From the above results it can be deduced that Mg hardly had any impact on the release of sugars or phenolics due to pretreatment of plant biomass. It seems that Mg has nothing to do with the structural integrity of the wood. On the other hand Zn and Ca seem to play some role in the structural configuration of the wood especially herb and shrub. In case of *A. exelsa* wood, apart from Zn and Ca, Fe was also found to have considerable impact on the release of sugars and thus all the three seem to play some role in the structural configuration of the tree wood.

Dilute acid pretreatment method was found suitable and economic earlier (15,16) and 0.7% acid was found to be optimum for pretreatment of *A. exelsa* wood (7,8).

***Effect of metal ions on the fermentation of treated H. suaveolens* wood hydrolysate :** The hemicelluloses hydrolysate from *H. suaveolens* wood was treated with lime and subjected to

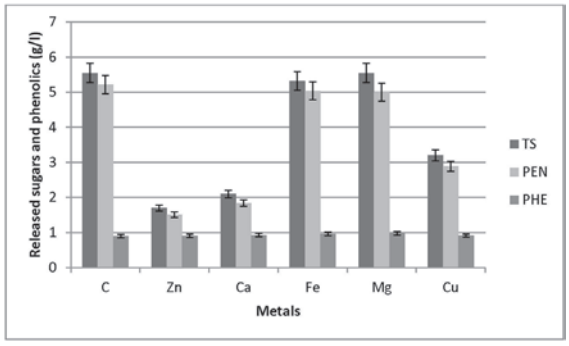

Fig. 1 Concentration of sugars and phenolics in the hydrolysate of *Hyptis suaveolens* wood released in presence of various tested metals

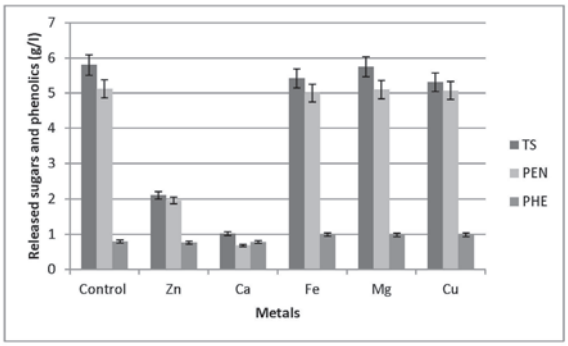

Fig. 2 Concentration of sugars and phenolics in the hydrolysate of wheat straw released in presence of various tested metals

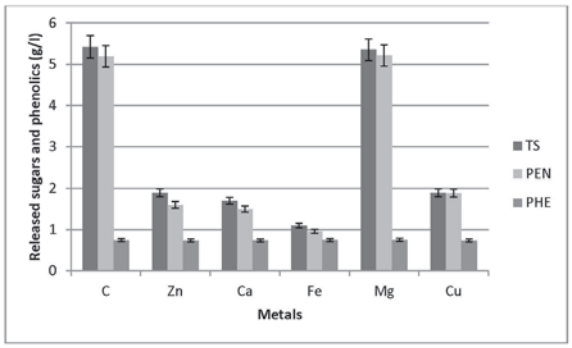

Fig. 3 Concentration of sugars and phenolics in the hydrolysate of *Ailanthus exelsa* wood released in presence of various tested metals (TS-total sugar; PEN-pentoses; PHE-phenolics)

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fermentation. There was a little impact on the utilization of sugars and production of ethanol (Fig. 4).

Effect of metal ions on the fermentation of treated wheat straw hydrolysate : The hemicelluloses hydrolysate from wheat straw was treated with lime and subjected to fermentation. Although there was little impact, negative effect of Cu and Fe on sugar utilization was evident, on the other hand Mg improved a little bit ethanol fermentation (Fig. 5).

Effect of metal ions on the fermentation of treated A. exelsa wood hydrolysate : The hemicelluloses hydrolysate from *A. exelsa* was treated with lime and subjected to fermentation. There was significant negative impact of all the metals on the sugar utilization by the yeast, ethanol fermentation was also inhibited a little bit by Cu (Fig. 6).

Metals present in the natural water were found in general to inhibit the release of hemicellulosic sugars and lignin. Metal ions were also found to interfere with sugar utilization and ethanol fermentation by the yeast. More specifically, Ca and Zn reduced the release of sugars in case of *H. suaveolens* wood by 62% and 70% respectively and in wheat straw by 82% and 64% respectively. In the case of *A. excelsa* wood, Ca, Zn, Fe and Cu prevented release of sugars by 68%, 63%, 80% and 63% respectively. The different response of woods from different source materials in presence of various metals indicates the compositional or configurational difference of woods. Ca and Zn seem to be general inhibitors of release of sugars during pretreatment by dilute acid method possibly through strengthening the configuration of the wood. Metals were also found to interfere with the fermentation of treated hydrolysate sugars. The result is important and highlights the importance of purity of water to achieve maximum efficiency pretreatment applying dilute acid method.

Conclusions

Ca and Zn are general inhibitors of the

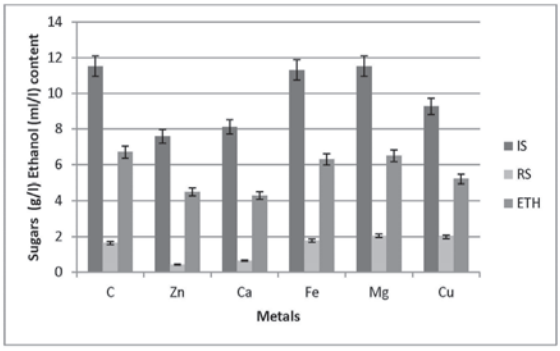

Fig. 4 Sugar utilization from hydrolysate obtained in presence of various metals and its subsequent fermentation to ethanol (IS- initial sugars before fermentation; RS-residual sugars after fermentation; ETH- ethanol)

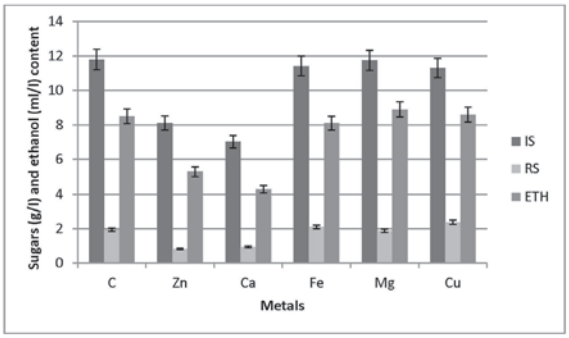

Fig. 5 Sugar utilization from hydrolysate obtained in presence of various metals and its subsequent fermentation to ethanol (IS- initial sugars before fermentation; RS-residual sugars after fermentation; ETH- ethanol)

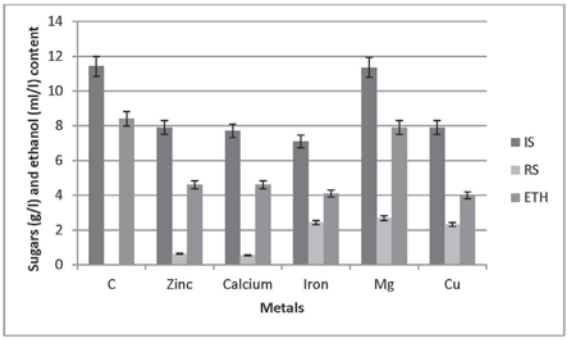

Fig. 6 Sugar utilization from hydrolysate obtained in presence of various metals and its subsequent fermentation to ethanol (IS- initial sugars before fermentation; RS-residual sugars after fermentation; ETH- ethanol)

release of hemicellulosic sugars. Mg has no effect. In case of tree wood Fe and Cu may also inhibit release of hemicellulosic sugars. It seems that structural integrity of various types of plants (herb, shrub and tree) depends on some metals as well. More works involving more types of plants are required to get a fuller appreciation of general role of metals on the efficiency of pretreatment of wood applying dilute acid or other methods.

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Overview of enzyme based biosensors and their applications

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Abstract

Enzymes are biocatalysts that govern life processes. Each biochemical reaction of cell metabolism is catalyzed by one specific enzyme. Enzymes can catalyze reactions in different states but most commercial enzymes, being water soluble are difficult to recover at the end of the catalytic process. This restricts the use of soluble enzymes to essentially batch-operations followed by disposal of these expensive enzymes containing solution. A possible approach to resolve this problem is to attach enzymes onto a solid/ semi solid support material. Such an attachment restricts the free movements of the enzyme molecules and renders them insoluble in aqueous media. The process of attachment of enzymes on an insoluble solid/semisolid material by physical or chemical bonding known as immobilization. In the present review we have discussed in brief about the enzyme-based biosensors, their history of development, classification and their applications in various filed with special emphasis on medicine and analytical assays.

Keywords: Enzymes, Biosensors, Biocatalyst, Immobilization

Introduction

Enzymes are powerful biological catalysts which serve to accelerate the chemical reactions of living cells. Without enzymes, most of the biochemical reactions would be too slow to even carry out life processes. Enzymes display great substrate selectivity and perform best at optimum pH and temperature. Besides their role as biocatalysts, enzymes are being increasingly used in industries, medicine and analysis of biomolecules (1-5).

Enzymes are not changed during the reactions, as they are soluble in reactants or products, so it is difficult to separate them. Therefore, if an enzyme can be attached (immobilized) to an insoluble support material by any means (physically or chemically), it can be used repeatedly after the products have been removed. Immobilization thus offers an effective way to enhanced stability and also the recovery of enzyme at the end of catalytic process, which improve activity and purity of products (6-11).

Every immobilization technique is not suitable for all enzymes. Hence, it is important to understand the physical and chemical changes in enzyme when it undergoes immobilization. Numerous factors can affect the rate of the enzyme's catalytic activity; changes have been observed in the stability and kinetic properties of enzyme due to the microenvironment and product's characteristics (12-13).

It is most important to choose a method of attachment which prevent loss of enzyme activity as well as it should not allow change in chemical nature and binding site of the enzyme. It is desired to avoid reaction with the essential binding site group of the enzyme. Alternatively, an active site can be protected during attachment as long as the protective groups can be removed later on without loss of enzyme activity (14-15).

The use of immobilized enzymes in industries, pharmaceutical companies and in biochemical analysis has increased tremendously

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in recent years (16-19). In this review, we discuss the basic principles of enzyme based biosensor, their history of development, classification and their applications in various filed with special emphasis on medicine and analytical assays.

Historical development of enzyme-based biosensors : The science and technology of immobilized enzymes has experienced a phenomenal growth in recent years (20-23). The very first report on the immobilization of proteins via adsorption of invertase on activated charcoal was reported by Nelson and Griffin (20), way back in 1916. The first enzyme electrode, an amperometric system, was described by L.C. Clark and C. Lyons (21) and S. J. Updike and G.P. Hicks (22), using glucose oxidase entrapped onto a polarographic oxygen electrode, for the measurement of glucose in biological solutions and tissues. In such amperometric or voltammetric probes, the current, produced upon application of a constant applied voltage, is measured. The first potentiometric enzyme electrode was described by G. Guilbault and J. Montalvo (23). The historical data of biosensor development are shown in Table1.

Methods of preparation of enzyme electrode based biosensors : Biosensor can be prepared in one of the four possible ways viz., membrane entrapment, physical adsorption, matrix entrapment and covalent bonding (36-37). In the membrane entrapment, a semi permeable membrane separates the analyzer and the enzyme, where the sensor is attached to the enzyme. The physical adsorption is dependent on a combination of van der Waals forces, hydrophobic forces, hydrogen bonds, and ionic

Table 1 Historical data of biosensor development

Year	Event	Reference
1916	First report on the immobilization of proteins: adsorption of invertase on activated charcoal	(20)
1922	First glass pH electrode	(24)
1956	Invention of the oxygen electrode	(25)
1962	First description of a biosensor: an amperometric enzyme electrode for glucose	(21)
1969	First potentiometric biosensor: urease immobilized on an ammonia electrode to detect urea	(26)
1970	Invention of the ion-selective field-effect transistor (ISFET)	(27)
1972-75	First commercial biosensor: Yellow Springs Instruments glucose biosensor	-
1975	Invention of the pO_2/ pCO_2	(28)
1976	First bedside artificial pancreas	(29)
1980	First fiber optic pH sensor for in vivo blood gases	(30)
1982	First fiber optic-based biosensor for glucose	(31)
1983	First surface plasmon resonance (SPR) immunosensor	(32)
1984	First mediated amperometric biosensor: ferrocene used with glucose oxidase for the detection of glucose	(33)
1987	Launch of the MediSense ExacTech blood glucose biosensor	-
1990	Launch of the Pharmacia BIACore SPR-based biosensor system	(34)
1998	Launch of Life Scan FastTake blood glucose biosensor	
1998	Merger of Roche and Boehringer Mannheim to form Roche Diagnostics	(35)

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forces to attach the enzyme to the surface of the sensor (36). The porous entrapment is based on forming a porous encapsulation matrix around the enzyme that helps in binding it to the sensor. In the case of the covalent bonding the sensor surface is treated as a reactive group to which the enzyme can bind.

To construct an enzyme electrode, the enzyme must react most selectively with the substance to be determined. It is important to check the purity of enzyme, availability and also the selectivity. The better the immobilization procedure and the more stable the enzyme; longer it can be used analytically. Generally the physically entrapped enzyme is stable for about 3 weeks or 100 assays and the chemically bound enzyme for over a year and up to 1000 assays (37).

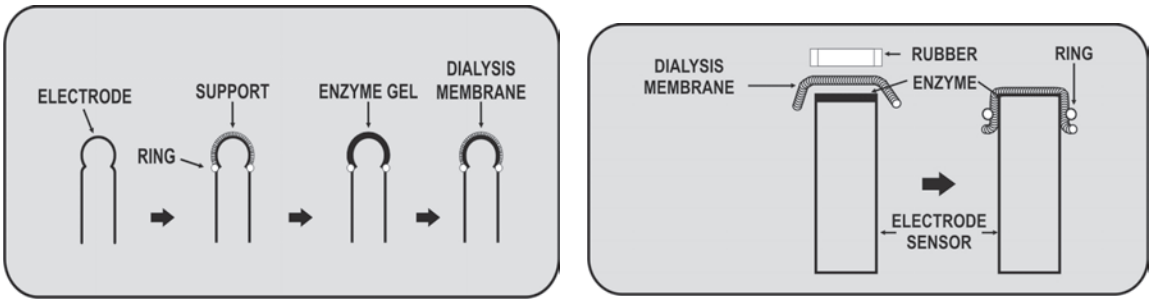
The electrode probe may be assembled as described by the diagram in (Figure-1) using configuration-A, for the physically entrapped enzymes and configuration-B for the chemically bound or soluble entrapped enzymes. The base sensor used is chosen according to the enzyme reaction to be studied. It must respond either to one of the products or to one of the reactants of the enzyme system.

Working of biosensor and its classification :

Biosensor is a device which is associated with the electronic signal processors that are primarily responsible for the display of the results in a user-friendly way. Biosensor obtains quantitative and semi-quantitative information using biological recognition element (biochemical receptor), which is in a space contact with a transducer (21). The readers are usually custom-designed and manufactured to suit the different working principles of biosensors (38). Enzyme based biosensor has large protein molecule that acts as a catalyst in chemical reactions, but remains unchanged at the end of reaction. An enzyme upon reaction with a substrate forms a complex molecule which under appropriate conditions forms the desirable product molecule releasing the enzyme at the end.

Biosensors may be classified according to a biological recognition material, a way of transformation of a physical–chemical signal into electrical one, by a transducer, a registered reaction or an analyzed substance.

Enzyme reactors can operate batch-wise or continuously. Batch processes with the enzymes usually hydrolyses in an aqueous reaction medium. Despite its numerous applications have several drawbacks, as enzymes are poorly stable and hard to recover in such systems, leading to



(A) (B)

Fig. 1. Different methods for preparation of enzyme electrodes based biosensors (configuration-A by Physical entrapment and configuration-B by Chemically attached enzyme)

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low productivity. Poor stability is usually the limiting factor in any enzyme process so that enzyme stabilization during reactor operation is a major concern (39, 40). Immobilized enzymes can be used in batch processes but in this case the enzyme is recovered to be used in subsequent batches until the accumulated inactivation makes necessary to replace the spent biocatalyst. As a consequence, specific productivity (mass of product/mass of biocatalyst time of operation) is increases and bioreactor design becomes flexible to suit the needs of a given process (41).

Types of biosensors

Resonant Biosensors : A resonant biosensor uses wireless modules for data transmission, communication and alarm. Photonic sensor technologies are advantageous as they are immune to outside electromagnetic interference, permit compact format and some types enable effective light input and output (42). The functioning of resonant biosensors based on fact that, the molecule (or antigen) gets attached to the membrane and the mass of the membrane changes. The resulting change in the mass subsequently changes the resonant frequency of the transducer (43).

Optical-detection Biosensors : The optical-detection biosensors device is based on detecting changes in absorption of a gold layer. In this device the changes in absorbance or fluorescence of an appropriate indicator compound and do not need a total internal reflection geometry. One of such example is, a fully operational prototype device detecting casein in milk has been fabricated. Another widely used biosensor is based on the micro-array analysis which could be proved to be a vital tool in research (44).

The biosensor also can be made based on optical diffraction or electrochemiluminescence, where transduced signal measures light. In optical diffraction based devices, a silicon wafer is coated with a protein via covalent bonds. The wafer is exposed to UV light through a photo-mask and the antibodies become inactive in the exposed regions. When the diced wafer chips are incubated in analytes, antigen-antibody bindings are formed in the active regions, thus creating a diffraction grating (45). This grating produces a diffraction signal when illuminated with a light source such as laser. The resulting signal can be measured or can be further amplified before measuring for improved sensitivity.

Thermal-detection Biosensors : These class of biosensors are coupled with temperature detectors. They are constructed by combining immobilized enzyme molecules with temperature sensors (46). When the analyte comes in contact with the enzyme, the heat of reaction of the enzyme is measured and is calibrated against the analyte concentration. The total heat produced or absorbed;is proportional to the molar enthalpy and the total number of molecules in the reaction. The measurement of the temperature is typically accomplished via a thermistor, and such devices are known as enzyme thermistors. Their high sensitivity to thermal changes makes thermistors ideal for such applications (46).

Ion-Sensitive Biosensors : Ion-sensitive sensors work on the principle that the interaction of ions with a semiconductor changes the electric potential of the semiconductor surface (47). The potential changes can then be measured to evaluate the desired parameter. Sensors with ion Sensitive Field Effect Transistor (ISFET) can be constructed by covering the sensor electrode with a polymer layer. This polymer layer is selectively permeable to 4 analyte ions. The ions diffuse through the polymer layer and in turn cause a change in the FET surface potential. This type of biosensor is also called an ENFET (Enzyme Field Effect Transistor) and is primarily used for pH detection (47).

Electrochemical Biosensors : Electrochemical Biosensors involves the generation of ions by various chemical events that change the electrical properties of the analyte solution (48). The concentration of the analyzer is then measured with respect to this change. Such biosensors are mainly used for the detection of hybridized DNA, DNA-binding drugs, glucose concentration, etc(49,

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50). A comparative discussion of different types of electrochemical biosensors is given in Table 2 (49-53).

Electrochemical Biosensors further can be classified based on the measuring electrical parameters such as: (1) conductimetric, (2) amperometric and (3) potentiometric.

Conductimetric : Conductometric biosensors are based on principle that when electrochemical reactions produce ions or electrons, the overall

Table 2. Different types of transducers used in biosensor construction

Transducer	Examples
Electrochemical	Clark electrode; mediated electrodes; ion-selective electrodes (ISEs); field-effect transistor (FET)-based devices; light addressable potentiometric sensors (LAPS)
Optical	Photodiodes; waveguide systems; integrated optical devices
Piezoelectric	Quartz crystals; surface acoustic wave (SAW) devices
Calorimetric	Thermistor; thermopile
Magnetic	Bead-based devices

Table. 3. Analytical application of enzyme based biosensors

Sr. No.	Analyte	Enzymes	Detection principle	Reference
1	Alanine aminotransferase	Pyruvate oxidase	Colorimetric	(55)
2	á-Amylase activity	Glucan 1,4-á-glucosidase + GOD	H_2O_2 or O_2	(56)
3	Urea	Urease	Cation electrode	(23)
4	Lactic acid	LDH + Cataiase	$Fe(CN)_6$ redox electrode	(57)
5	Galactose	Galactose oxidase	H_2O_2	(58)
6	Glucose	GOD	O_2	(22)
7	Phenol	Tyrosinase	O_2	(59)
8	Glutamine	Glutaminase	pH	(60)
9	Lactate/glucose	LOD/GOD	O_2	(61)
10	Cholesterol esters	Cholesterol esterase/ oxidase	H_2O_2	(62)
11	Ascorbic acid	Ascorbic acid oxidase	Thermistors	(63)
12	Sucrose	Invertase	Thermistors	(64)
13	Sucrose	Invertase, Mutarotase, GOD	O_2	(65)
14	Maltose	Amyloglucosidase + GOD	H_2O_2	(66)
15	Sucrose	Invertase, Mutarotase, GOD	O_2	(67)
16	Amino acids	Amino acids oxidase	O_2	(68)
17	Phosphate	Acid phosphatase + GOD	O_2	(69)
18	Carboxylic acid	Alcohol oxidase	O_2	(70)
19	Penicillin	Penicillinase	H^+	(71)
20	Glucose	GOD	Optic fiber	(72)

Overview of enzyme based biosensors and their applications

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conductivity or resistivity of the solution changes. This change is measured and calibrated to a proper scale. Conductance measurements have relatively low sensitivity. The electric field is generated using a sinusoidal voltage (AC) which helps in minimizing undesirable effects such as Faradaic processes, double layer charging and concentration polarization.

Amperometric : Amperometric transducers measure the flux of the electroactive species they are set up to detect. They have been used most widely to determine the decrease in oxygen tension or the increase of product (H_2O_2) in enzymatic oxidation-reduction reactions (51, 52).

Potentiometric: Potentiometric biosensors are generally chosen so as to detect the product of the enzyme reaction. In particular, pH electrodes and ammonium ion selective electrodes have been used for hydrolytic enzyme based electrodes (53,54).

Applications of biosensors in various fields

The commercial value of enzymes is linked to their applications as process catalyst and various medical fields (i.e. medical diagnostics, in-vitro and in-vivo diagnostics etc.) as described below (22, 56-72).

Biosensors for medical diagnostics*:-* Biosensors have wide range of application, the most important application is in the field of medical diagnostics for both In vivo and in vitro .

In vitro diagnostics*:-* In vitro medical diagnostics has a worldwide good market income but this required strict pre-market approval for these diagnostic tests. These diagnostic tests fall in following categories.

Centralized tests in hospitals*:-* These tests conducted in hospitals include tests for glucose, lactate, uric acid, viruses, and a variety of pathogenic microbes.

Tests in doctors clinics*:-* Analysers (in the form of portable biosensors) to be used in the nursing homes or in private clinics of practicing doctors, are also being developed for testing glucose, lactate, creatinine and urea.

Analytical application of biosensors:- The analytical application of enzyme based biosensors are receiving increasing attention as it has a direct role in various fields and majorly in medical field (22, 56-72). For the determination of a specific analyte, choice of enzymes and detection principle are compiled in Table 3, which give easier access to the various procedures and the choice of method to serve as a starting point of detection of analytical assays for a given analyte.

Conclusion

Immobilization of enzymes improves stability of enzymes. Recent use of immobilized enzymes in analytical applications is enjoying tremendous popularity. Today's biosensor market is dominated by glucose biosensors and pregnancy kits. Here in the present review we have tried to look into the historical background, classification, growth and development of the enzyme biosensor field from a strong commercial viewpoint. The current status of the technology is evaluated and future trends in this dynamic and fast-moving field are also anticipated.

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NEWS ITEM

IIT Delhi researchers find out novel ways to assess bacterial drug sensitivity

A two-member team from the Indian Institute of Technology (IIT) Delhi has used a novel method to culture bacteria and determine its growth at much lower concentration in relatively less time — four–six hours. E. coli and S. aureus bacteria were studied. Currently available clinical methods require more than 10 hours to culture and observe growth of pathogenic bacteria and a higher bacterial concentration for laboratory confirmation. While the new method will not be useful in identifying the species of bacteria isolated from a patient sample, it will help in early detection of the presence of bacteria and carrying out drug susceptibility testing within a short time period.

IACS researchers developed novel anti cancer compound

Researchers at Kolkata's Indian Association for the Cultivation of Science (IACS) have synthesised a novel compound that shows potent anticancer activity. The porphyrin compound selectively targets and binds to cellular topoisomerase 1 enzyme (which is essential for maintenance of DNA architecture in the cells) found in cancer cells. Since Top1 enzyme is essential for cell replication and transcription from DNA to RNA, inhibition of its activity leads to DNA torsional strain, overproduction of reactive oxygen species, degradation of the DNA and ultimately cell death.Though the currently available drug and its derivatives and the compound synthesised by IACS researchers target Top1 enzyme, the pathways are very different, which was validated in both in vitro and ex vivo cellular studies. As a result, even when chemoresistance to currently available drug (camptothecin) sets in, the new compound could be used for effective killing of cancer cells

IIT- Delhi, AIIMS duo devised a novel apparatus to combat air pollution

The AIIMS in collaboration with the researchers of the Indian Institute of Technology (IIT), Delhi have developed a wearable nasal device which restricts the entry of air pollutants into lungs. Named 'Airlens', the two cm device has the capacity to trap pollutants and bring them to safe levels depending on the air quality outside. The use and throw device, which is awaiting a patent, does not need to be attached to anything. In the beginning, the device would be for children aged above 6 years. India had the world's highest number of deaths due to air, water and other forms of pollution in 2015, according to a study published in the Lancet journal last week.

DRDO designed a novel ayurvedic drug that could relieve Vitiligo

Vitiligo or Leucoderma is a skin condition, which affects close to 2-5 percent of the population in India. Vitiligo is not usually medically harmful, and is non-contagious, but can cause white spots or patches to appear on the skin. DRDO scientists have developed Lukoskin, an Ayurvedic drug, and claims that it can greatly ease vitiligo. Allopathic medicines mask the disease, and the patient feels that he/she is healed, but it is not so. Once the patient stops taking the medicine, there is a relapse. However, this is not the case with herb-based medicines. Lukoskin was initially developed in 2012, following many comprehensive research and clinical trials by scientists of the Defence Bio-Energy Research (DIBER) centre at Haldwani, Uttarakhand.Comprising of an ointment and an oral liquid, patients consume the drug over a period of 300 to 400 days for effective results. AIMIL Pharmaceuticals in Delhi have been given the authority to manufacture the drug.

IGIB scientists identified Protein regulating both skin cancer and pigmentation

Researchers at Delhi's CSIR-Institute of Genomics and Integrative Biology (IGIB) have for the first time identified a calcium sensor protein (STIM1) that independently regulates both skin cancer and pigmentation. The STIM1 protein does so by activating two independent signalling

pathways. Interestingly, different parts of the STIM1 protein activate the two independent signalling pathways that control melanoma growth and pigmentation. The role of STIM1 in breast cancer and prostrate cancer is already known. To study the role of STIM1 protein in melanoma growth in vitro, the researchers used STIM1 knockdown mouse cells and injected them into mouse models and observed the growth of melanoma. Compared with controls, melanoma growth was reduced by as much as 75% in mice that were injected with STIM1 knockdown cells. Similarly, melanin level reduced when pigmentation decreased. A surprising finding was that when pigmentation was decreasing, the calcium signalling pathway was also decreasing. The paper published in The EMBO Journal.

18 novel species of Madagascan spiders unearthed

Eighteen new species of Madagascan spiders have been discovered, a report published on January 11 in the science journal Zookeys reveals. According to a release from Smithsonian Institution, the discovery adds to scientists' understanding of the biodiversity that exists in Madagascar, It also highlights the need for conserving what remains of Madagascar's forests, what with widespread deforestation posing a threat. Native to Madagascar, these spiders are also called as "living fossils," as similar ones have been found in 165-million-year-old fossils.

Turmeric, a Hub of anti cancer drugs, says researchers from UoH

A research finding by the University of Hyderabad (UoH) might finally pave the way in developing a drug against cancer. Although turmeric has been used since ancient times as home remedy for various ailments in India, and modern medical science has already established the curative properties of the compound 'curcumin' found in the yellow spice, researchers have not been able to effectively utilise the same. This is because of the low solubility, half-life and bio-availability of curcumin in the form of a drug. Artemisinin is also a plant derived compound. For a drug to be effective in human body against a disease, it is essential that it is easily soluble in the blood/plasma and stays long enough in circulation in the body in order to be potent — bio-availability. This has been a major hurdle till now as pure curcumin is neither soluble nor does it stay long enough in the body. However, UoH researchers found that when CUR-ART co-amorphous in solid form was administered orally to mice at a dosage of 200 milligrams per kilogram (mg/Kg) of body weight, solubility levels of 0.90 - 1.23 microgram per milliliter of blood was recorded in 30 minutes.

IICB reveals molecular basis of gastric ulcers induced by stress

Researchers at Kolkata's CSIR-Indian Institute of Chemical Biology (CSIR-IICB) have for the first time identified the molecular mechanism by which acute mental stress affects the stomach causing gastric ulcer or stress-related mucosal disease. Using a rat model subjected to cold-restrained stress the Division of Infectious Diseases and Immunology at IICB has used drugs that can act specifically on mitochondria present in the stomach to prevent gastric ulcer caused by stress.When subjected to stress, the mitochondrial respiratory capacity was disrupted, ATP production was reduced and oxidative stress increased. Stress also causes morphological changes to the mitochondria such as increased fragmentation. The results of the study were published in the journal Free Radical Biology and Medicine. Due to oxidative stress and fragmentation, the mitochondria in the gastric mucosal lining cannot behave in a normal fashion and ATP production gets further compromised. In the absence of ATP production, cells cannot proliferate and the gastric lining gets thinner due to mucosal cell death.

Researchers from IISc demonstrates the social division of insects, first of its kind

Using an Indian paper wasp, researchers at Benguluru's Indian Institute of Science (IISc) have for the first time been able to witness in laboratory settings the minimum conditions

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required for the emergence of cooperation and division of labour — important for evolutionary success and ecological dominance — among social insects. How the two important features influence productivity (total brood of the colony) was already known theoretically but not adequately demonstrated empirically till now.Using newborn virgin female wasps (Ropalidia marginata) the researchers demonstrated the spontaneous emergence of cooperation and two types of division of labour — reproductive and non-reproductive. The reproductive division of labour determines who becomes the queen and reproduces, and who becomes the worker and carries out tasks other than reproduction. When more than two workers are present in a nest, an additional division of labour emerges that determines who does the housekeeping job and who does work outside the nest.

Chinese scientists overcomes technical hurdles to clone monkeys

Researchers at the Institute of Neuroscience in Shanghai have cloned monkeys using the same technique that produced Dolly the sheep two decades ago, breaking a technical barrier that could open the door to copying humans. Zhong Zhong and Hua Hua, two identical long-tailed macaques, were born eight and six weeks ago, making them the first primates — the order of mammals that includes monkeys, apes and humans — to be cloned from a non-embryonic cell.It was achieved through a process called somatic cell nuclear transfer (SCNT), which involves transferring the nucleus of a cell, which includes its DNA, into an egg which has had its nucleus removed. Researchers said their work should be a boon to medical research by making it possible to study diseases in populations of genetically uniform monkeys. But it also brings the feasibility of cloning to the doorstep of our own species. The new research, published in the journal Cell, shows that is not the case. The Chinese team succeeded, after many attempts, by using modulators to switch on or off certain genes that were inhibiting embryo development.

MIT researchers discovered a novel virus from oceans

Researchers at the Massachusetts Institute of Technology and the Albert Einstein College of Medicine have reported a new tailless virus prevalent in the world's oceans. These viruses remained undiscovered till now as they cannot be detected using standard tests. The new find was made possible by novel genomic studies, and scientists say these viruses could be the missing link in the evolution of viruses. The virus has been named after a character in Greek mythology, Autolykos, who was a trickster and difficult to catch.The study published in Nature shows that these viruses mainly feed on bacteria, and could be helping in regulating the bacterial populations of the ocean. Every drop of surface ocean water can contain almost ten million viruses.

Mosquitoes can differentiate the host's odors

Mosquitoes can rapidly learn and remember the smells of hosts, a study suggests. Dopamine is a key mediator of this process. Hosts who swat at mosquitoes or perform other defensive behaviours may be abandoned, no matter how sweet they are, according to the study published in Current Biology. Mosquitoes develop preferences for a particular vertebrate host species, and, within that population, certain individuals, they said. However, the study also proved that even if an individual is deemed delicious-smelling, a mosquito's preference can shift if that person's smell is associated with an unpleasant sensation. The researchers said mosquitoes exhibit a trait known as aversive learning by training female aedes aegypti mosquitoes to associate odours with unpleasant shocks and vibrations. Twenty-four hours later, the same mosquitoes were assessed in a Y-maze olfactometer in which they had to fly upwind and choose between the once-preferred human body odour and a control odour. The mosquitoes avoided the human body odour, suggesting that they had been successfully trained.

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SCIENTIFIC NEWS

PSLV – C40 successfully puts in 31 satellites into orbits

The 42nd Polar Satellite Launch Vehicle (PSLV), PSLV-C40, was launched successfully by the Indian Space Research Organisation (ISRO) from the First Launch Pad of the Satish Dhawan Space Centre (SDSC) in Sriharikota and it placed 31 satellites across two orbits.The PSLV, launched at 9.29 a.m., had as its primary payload the country's fourth satellite in the remote sensing Cartosat-2 series, weighing 710 kg. The 30 other co-passenger smaller satellites, together weigh 613 kg. Of them, 28 are from other countries.The Cartosat-2, whose imagery will be used to develop various land and geographical information system applications.

Significant levels of Delhi smog amounting to 40% was dust emanating from Gulf region

Dust travelling thousands of kilometre from a severe storm in the Gulf contributed significantly to the weeklong killer smog that choked Delhi-NCR and much of north India from November 7 onwards, an analysis by the government's air quality research body, SAFAR, has concluded. SAFAR said dust coming in from the Gulf constituted nearly 40% of pollutants in the smog while stubble-burning in Punjab and Haryana contributed around 25%. Making up the remaining 35% was pollution produced locally in Delhi-NCR+ .In a nightmarish confluence of factors, favourable upper winds carried the dust from the Gulf and smoke from crop burning into Delhi-NCR while an anti-cyclonic wind circulation over the region pushed these pollutants towards the surface and trapped them there as surface conditions were calm.PM 2.5 concentration was recorded at 537 micrograms (g)/m3 on November 7, nine times the 24-hour average standard. It rose to a peak of 640 g/m3 the next day. According to the SAFAR analysis, if external sources of pollution had not played a role, Delhi's air quality on November 8 would have been closer to 200 g/m3.

Study shows strict Social norms may imprint genes

A study by researchers from the National Institute of BioMedical Genomics (NIBMG) in West Bengal has looked at the genes of various communities to answer questions that have often been suggested in history books: when did caste become the dominant norm for ethnic communities of the region.For most upper-caste communities, endogamy (that is marrying within one's caste) started nearly 70 generations ago, or around the time of the Hindu Gupta period around 1,500 years ago, says the study published in the latest issue of the journal PNAS (Proceedings of the National Academy of Sciences of the United States of America).

IUCN new Red data book unleashes extinct species

According to the updated IUCN (International Union for Conservation of Nature) red list, species Christmas Island Pipistrelle, Christmas Island Whiptail-skink and Gunthers Dwarf Burrowing skink that were marked as "EXTINCT". The new updated list also figure out critically endangered and vulnerable categories.

Earth going to witness Super Moon, Blue Moon and a total lunar eclipse

Get ready for a rare lunar event that has kept the Internet abuzz from the beginning of the year. A Super Moon, Blue Moon and a total lunar eclipse can be seen on the evening of January 31 2018. During a total lunar eclipse, though the Moon gets shadowed by the Earth, sunlight passing through the Earth's atmosphere, break down in its constituent colours and the red part gets scattered by the atmosphere and falls on the Moon's surface, thereby making it take on a reddish copper hue. For this reason since antiquity, a totally eclipsed Moon is called a Blood Moon.

Obituary :

Prof Lalji Singh, Father of DNA fingerprints passes away. Prof Lalji Singh held the position of BHU VC from August 22, 2011 to August 22, 2014.

He is considered to be father of DNA fingerprints, who worked in the area of molecular basis of sex determination, wildlife conservation, forensics and evolution and migration of humans. He also served as director of the Centre for Cellular and Molecular Biology (CCMB) apart from assuming other higher positions.

POST DOC OPPORTUNITIES:

1. NCBS-inStem-Cambridge Postdoctoral Fellowship (NiC-PDF) : The National Centre for Biological Sciences (NCBS), Bangalore, the Institute for Stem Cell Biology and Regenerative Medicine (inStem), Bangalore, and the University of Cambridge **http://www.cam.ac.uk/** (UoC) are pleased to announce the NCBS-inStem-Cambridge Postdoctoral Fellowship (NiC-PDF).

2. National Post Doctoral Fellowship : The SERB-National Post Doctoral Fellowship (N-PDF) is aimed to identify motivated young researchers and provide them support for doing research in frontier areas of science and engineering. The fellows will work under a mentor, and it is hoped that this training will provide them a platform to develop as an independent researcher. For further information visit **http://www.serb.gov.in/npdf.php**

3. Fellowships of Ministry of Science & Technology (DST/DBT/CSIR(DSIR)/SERB) : Ministry of Science & Technology provides fellowships of different categories ranging from J C Bose National Fellowship to Ramanujan Fellowship and others, please visit for further information visit **http://www.dst.gov.in/fellowship-opportunities-researchers**

4. IISER Pune Postdoctoral Research Associate : Applications are invited for Postdoctoral Research Associate (PRAs) positions at the Indian Institute of Science Education and Research (IISER) Pune, India. These positions are open for candidates with 0-5 years of experience after the submission of their PhD thesis. For further information visit **http://www.iiserpune.ac.in/links/postdoctoral-research**

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