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Acute Hypobaric Hypoxia Induced Early Phase Biochemical and Histological Changes in Susceptible and Tolerant Rat Lung Tissues

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Abstract

Aim: Oxidative stress during early phase of acute hypobaric hypoxia may predispose an individual susceptible to critical altitude illness while ascending rapidly to high altitudes. We assessed the biochemical parameters and examined the histological changes to check the oxidative stress status of the lung tissues of Sprague-Dawley rats, susceptible and tolerant to acute hypobaric hypoxia during early phase of exposure.

Methods: A simulated acute hypobaric hypoxia of one hour at 9144 m and 24°C was given to susceptible male, normal male (moderate), tolerant male and female groups of rats. Reactive oxygen species (ROS) levels, malondialdehdye (MDA) and oxidized glutathione (GSSG) content, total catalase (CAT) activity, superoxide dismutase (SOD) inhibitory activity and glutathione peroxidase (Gpx) activity were measured using standard protocols in lung tissue samples isolated from the different groups of hypoxia stressed and normoxic rats. Histological changes were also studied by haematoxylin and eosin staining in these tissue samples.

Results: Marked neutrophil infiltration, alveolar wall collapse and interstitium thickening was visible in susceptible lung tissues showing maximum ROS levels, MDA equivalents and GSSG activity comparative to other test samples. Although SOD inhibitory activity increased in susceptible and normal groups in comparison to both the tolerant groups, however, Gpx activity showed an opposite trend. Catalase activity was recorded highest in the normal rat group as compared to other groups.

Conclusion : Increased neutrophil infiltration coupled with higher oxidant's levels in susceptible rat lungs, and increased antioxidant enzyme activity in normal and tolerant rats demonstrate the differential physiological states in lung tissues, which could be diagnosed even in early phase of acute hypobaric hypoxia stress.

Key words hypobaric hypoxia, high altitude, antioxidants, oxidative stress, Sprague-Dawley

Introduction

Mountaineers ascending rapidly to high altitudes develop acute hypobaric hypoxia due to severe decrease in partial pressure of oxygen in atmosphere. As the oxygen gradient falls, oxygen intake by lungs decreases creating oxidative stress in alveoli. During normal respiration, Complex I and III of electron transport chain in mitochondria release reactive oxygen species (ROS) generated as by-products during the serial electron transfer to the terminal acceptor, oxygen, in order to form water molecule and generate ATP (1). These ROS are capable of oxidizing and damaging the cellular lipids,

proteins and DNA by initiating a cascade of reactions. However, their cellular levels are kept under control by the production of their scavengers or antioxidants, whose levels significantly rise during oxidative stress in order to maintain the steady state of homeostasis. When this redox balance is disturbed, excess ROS production may lead to lipid peroxidation in cell membranes out bursting into several peroxyl radicals and formation of a prominent mutagen, malondialdehyde (MDA), which has long been employed as a biomarker of lipid peroxidation (2). The antioxidant enzyme, superoxide dismutase(s) (Sod1, Sod2 and Sod3) localized in various organelles and cytoplasm of the cells as well as the extracellular spaces convert the superoxides into hydrogen peroxide (3). Although, hydrogen peroxide is also an ROS and a signaling molecule, its levels are kept in control by antioxidant enzymes such as catalase found in the peroxisomes and glutathione peroxidases having many isoforms reported in mammals. In the subsequent reaction, Gpx is oxidized by H_2O_2 and reversibly reduced by glutathione.

Inside a normal cell, the ratio of GSH (reduced form) to GSSG (oxidized glutathione) is regulated such that small amount of GSSG is found owing to its enzymatic reduction and *de novo* synthesis of its reduced form (4). However, the higher amount of GSSG lowers this ratio during oxidative stress and drives the cells towards apoptosis due to unfolding protein response and disruption of the integrity of nuclear components, which otherwise thrive and function in a reduced environment. The apoptotic cells are engulfed by alveolar macrophages, the depletion of which results in the acute inflammation following marked neutrophil infiltration and activation, when the alveolar-capillary barrier is broken during acute lung hypoxia (5). Acute hypobaric hypoxia, therefore, may lead to the development of critical high altitude illness such as Acute Mountain Sickness (AMS) and High Altitude Pulmonary Edema (HAPE) in lungs. However, an individual may be genetically predisposed to susceptibility or tolerance to these altitude disorders, as pathophysiological changes in lung tissues may show the variable extent of oxidative stress recognizable during early phase of hypobaric hypoxia exposure. We, therefore, assessed the oxidative stress status by studying biochemical parameters and histopathogical changes during early phase of hypoxia exposure to the lungs of susceptible and tolerant rats.

Materials and methods

Animal maintenance and ethical clearance: Sprague-Dawley rats (both male and female) were provided standard nutritional pelletized diet with sterile water daily and hygienically maintained at 24±2ºC with 12 hours routine cycles of day and night in the animal house facility of Defence Institute of Physiology and Allied Sciences (DIPAS), Defence Research Development Organisation (DRDO), New Delhi. These conditions and the protocol of providing simulated acute hypobaric hypoxia exposure to animals were in compliance with all the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and were approved by the Institute's Animal Ethical Committee, DIPAS, DRDO.

Screening of rats for group segregation : All the rats weighing 180±10 g were subjected to simulated altitude of 9754 m at 32°C and 205.6 mm Hg of atmospheric pressure, with 914.4 m per minute rate of decrease inside a decompression chamber (Decibel Instruments, India) till the arrival of first gasping. Based on this gasping time, four groups with their respective gasping time were identified namely: susceptible [Sus(M)] with < 5 minutes; normal [Nor(M)] between 15-20 minutes; tolerant male [Tol(M)] with > 45 minutes and tolerant female [Tol(F)] rats with > 1 hour gasping time. This protocol has been followed earlier in our studies on acute hypobaric hypoxia (6,7,8).

Simulation of acute hypobaric hypoxia :Above groups of rats were subjected to acute hypobaric hypoxia stress by exposure to a simulated altitude

of 9144 m (at 225.6 mm Hg) inside the decompression chamber for 1 hour at 24°C with relative humidity 40-50% maintained with airflow 2 l/min. Normoxic male [Nor(M)] and female [Nor(F)] rats were kept as control without any hypoxia treatment. After anaesthetizing with ketamine (80 mg kg^{-1} body weight, *i.p.*) and xylazine (20 mg kg^{-1} body weight, *i.p.*), animals were perfused using a blunt-tipped 20-gauge perfusion needle, inserted into the left ventricle of heart (9). The blood was allowed to flow out by incising right atrium for perfusion using the Phosphate Buffer Saline (PBS) pH 7.2. The lungs were removed, quickly immersed and washed repeatedly using PBS. These samples were submerged into liquid nitrogen and thereafter preserved at -80°C for antioxidant assays.

Histopathological studies : The haematoxylin and eosin stained slides of lung tissues were studied under a compound microscope to visualize the hypoxia induced histological changes. For lung tissue fixation, perfusion with 4% paraformaldehyde fixative solution was performed after perfusion with PBS. The lung tissue samples thus obtained were immersed into 10% neutral buffered formalin and kept for 48 hours at room temperature for proper fixation. The tissues dehydrated with increasing concentration of ethanol were submerged into 100% xylene solution and hardened by embedding in the heated paraffin wax. The paraffin embedded tissue blocks were finely sectioned using a microtome, melted and kept in 100% xylene. These slides were rinsed with water and stained with haematoxylin for 5 min, washed and quickly dipped for few times in 0.5% aqueous HCl. In a similar manner, 5% eosin was used to stain slides for few minutes with quick dips into increasing concentration of ethanol (95% and 100%) followed by xylene.

Estimation of biochemical parameters : The assays mentioned below were performed to examine the oxidant and antioxidant levels to estimate the oxidation status of the biochemicals such as lipids, proteins and DNA inside the lung tissue of animals exposed to hypobaric hypoxia.

The total protein content in each lung tissue sample was estimated using Bradford's assay by first obtaining the standard curve using different known concentrations of Bovine Serum Albumin (BSA) protein prepared in phosphate buffer saline at 595 nm.

Determination of reactive oxygen species : For measuring reactive oxygen species (ROS) content in the tissue sample, 100 μM 2'-, 7'-dichlorohydrofluoroscein diacetate was added to the supernatant obtained from the tissue homogenate (20 μg in 0.1 M PBS pH 7.4), and centrifuged at 10000 rpm for 10 min in the dark. The fluorescence intensity was read on excitation at 485 nm and emission at 530 nm in Fluorstar Optima (BMG Labtak, Germany). The amount of dichlorofuorescein (DCF) formed as a product was calculated as nmol min^{-1} mg^{-1} of protein directly proportional to the ROS content.

Malondialdehyde (MDA) estimation : The QuantiChrom TBARS assay kit from BioAssay Systems was used for estimating total malondialdehyde (MDA) that reacts with thiobarbituric acid (TBA) to form a pink coloured substance. The tissue samples in ice-cold PBS containing protease inhibitors were sonicated for 20 seconds at 40 volt. 200 μl ice cold 10% TCA was added to the 100 μl of each sample and the sample was incubated for 15 minutes on ice. It was then centrifuged for 5 min at 14,000 rpm. 200 μl of each sample supernatant was added to 200 μl TBA reagent, vortexed and incubated at 100°C for 60 min. About 100 μl from each tube in triplicates was loaded to the individual wells on Grenier 96 flat bottom assay plate and absorbance was read at 535 nm in Fluorstar Optima (BMG Labtak, Germany). The absorbance of blank was subtracted from O.D. values of all standard and sample values. The concentrations of all MDA standards were plotted against their average O.D. values obtained to determine the slope of the standard curve. The concentration of thiobarbituric acid reactive substances (TBARS) of each test sample was calculated as:

Concentration of TBARS (in μM MDA equivalents) = $[A_{Sample} - A_{Blank}] \times n$ /slope μM^{-1},

where A_{Sample} and A_{Blank} are the OD_{535} nm values of the sample and distilled water blank and *n* is the sample dilution factor.

Comparative oxidised glutathione level estimation : 20 µl of lung tissue homogenate in 100 mM solution was centrifuged at 10000 rpm for 15 minutes to extract the supernatant. 200 µl of 20% TCA was added to it and again centrifuged at 10000 rpm for 15 minutes to collect the supernatant. One third of N-methyl malemide (NEM) was added to it and incubated for 30 minutes at 37°C followed by addition of 1 ml of 0.1N NaOH. To an aliquot of 0.1 ml of this solutions 1.8 ml of 0.1N NaOH and 0.1 ml of 1 mg/ml o-phthaldialdehyde (OPT) solution were added and incubated for 15 minutes at room temperature. The excitation spectrum was recorded at 350 nm and emission at 420 nm in a fluoro-spectrophotometer Fluorstar Optima (BMG Labtak, Germany).

Estimation of antioxidant activity of selected enzymes

Superoxide dismutase assay : The working standard test solution 1 provided in the superoxide dismutase (SOD) activity assay kit from Biovision Inc. could be reduced by superoxide ion into a water-soluble formazan dye, the reaction rate of which is linearly related to the xanthine oxidase (XO) activity. Therefore, activity of SOD to inhibit this reaction was determined by a colorimetric method at 450 nm using a microplate reader. Tissue homogenised with 100 mM PBS was washed with same buffer to remove any red blood cells. Tissue was homogenized in ice cold 0.1M Tris/HCl, pH 7.4 containing 0.5 % Triton X-100, 5 mM β-ME, 0.1 mg/ml PMSF. The crude tissue homogenate was centrifuged at 14000 x g for 5 minutes at 4°C. The supernatant containing the total SOD activity was added to the wells of Grenier 96 flat bottom assay plate. The plate was incubated at 37°C for 20 minutes before recording the absorbance at 450 nm in Fluorstar Optima (BMG Labtak, Germany) to calculate percentage inhibition rate and the corresponding SOD activity in U ml^{-1}.

Catalase assay : The catalase activity was measured by using Cayman Chemicals Company kit that utilizes the peroxidase activity of catalase in the presence of hydrogen peroxide to convert methanol into formaldehyde. Formaldehyde forms a colourless complex with purpald (chromogen) that is oxidized to give purple colored product to be detected spectrophotometrically at 540 nm. For this assay, the lung tissue was homogenised in 5-10 ml of cold buffer (i.e., 50 mM potassium phosphate pH 7.0, containing 1 mM EDTA per gram tissue) and centrifuged at 10000 x g for 15 minutes at 4°C to remove supernatant. The samples for formaldehyde standard and positive controls were prepared as directed in kit protocol. The test sample wells were prepared by adding 100 µl diluted assay buffer, 30 µl methanol and 20 µl of the supernatant. To start the reaction, 20 µl of diluted hydrogen peroxide was added to all the wells. The time of the beginning of the reaction was noted and plate incubated on a shaker for 20 minutes at room temperature. 30 µl of diluted potassium hydroxide was added to each well to terminate the reaction followed by 30 µl of catalase purpald (chromogen). Plate was incubated for 10 minutes at room temperature and 10 µl of catalase potassium periodate was added to each well with incubation of five minutes at room temperature. The average absorbance of each standard and sample was recorded at 540 nm using a plate reader Fluorstar Optima (BMG Labtak, Germany) and subtracted from all other standards and samples. The formaldehyde concentration of the samples was calculated from the linear regression of the standard curve substituting corrected absorbance values for each sample.

Formaldehyde (µM) = [sample absorbance - (y-intercept)]/slope

The CAT activity of the sample was calculated from the following equation:

CAT Activity (nmol min^{-1} ml^{-1}) = (µM of sample/ 20 min) x sample dilution

Glutathione peroxidase assay : Conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG) by glutathione peroxidase (Gpx) with reduction of lipid hydroperoxides to their corresponding alcohols or free hydrogen peroxide to water was utilized in total glutathione peroxidase activity assay kit (Biovsion Inc). The generated GSSG was reduced to GSH with consumption of NADPH by the glutathione reductase (GR). Decreasing level of NADPH (measured at 340 nm) was proportional to the GPx activity. 100 mg tissue samples were homogenized on ice in 0.2 ml cold assay buffer and centrifuged at 10000 x g for 15 min at 4°C. The supernatant was collected for assay and stored on ice. 25 µl of the 40 mM NADPH solution was added to 975 µl dH_2O to generate 1 mM NADPH standard. The final volume was brought to 100 µl with assay buffer. The absorbance was measured at 340 nm for obtaining the NADPH standard curve. 5 - 10 µl GPx was added as positive control into the desired well(s) and adjusted to 50 µl with assay buffer and 50 µl of assay buffer was added into a well as a reagent control. For each well, 40 µl reaction mix was added to 33 µl assay buffer, 3 µl 40 mM NADPH solution, 2 µl GR solution and 2 µl GSH solution. 40 µl of the reaction mix was added to each test sample and positive control, incubated for 15 minutes to deplete all GSSG in the sample**.** 10 µl cumene hydroperoxide solution was added to start GPx reaction and the OD 340 nm was measured at one time point to read absorbance in Fluorstar Optima (BMG Labtak, Germany). The absorbance was also read at OD 340 nm again at second time point after incubating the reaction at 25°C for 5 min in the dark to calculate the difference.

Statistical analysis of biochemical assays: The mean values with their standard deviations (n = 6) obtained in all the six assays performed to determine ROS levels (nmol min^{-1} mg^{-1}), MDA equivalents (µM), GSSG levels (µM mg^{-1}), SOD activity (U ml^{-1}), CAT activity (nmol m^{-1} ml^{-1}), and GPx activity (mU ml^{-1}) were used to check the statistical significance of the results obtained using the software Graphpad Prism version 6.00 for windows. One way ANOVA and F test were used to test check whether there existed any statistically significant difference in these mean values when all male rat groups were compared (null hypothesis rejected any such differences). Barlett's statistic (corrected) was also included to check the significant variation in standard deviation (null hypothesis stating that there is no such difference). For evaluation of the variation in mean values due to gender difference, two way ANOVA and F test were used in order to compare control male, control female, tolerant male and female groups with each other. Since GSSG levels were calculated with respect to control levels in each case, therefore, t test (unpaired and two-tailed) was used to compare two sets i.e. Tol(M) vs Con(M), and Tol(F) vs Con(F).

Results

Histological studies : The control lung tissue sample showed Type I and Type II alveolar epithelial cells and tissue macrophages at certain places as shown in figure 1a, while susceptible and normal samples showed absence of alveolar macrophages in the luminal spaces (figures 1b and 1c) and susceptible sample rather showed aggregates of neutrophils in the interstitial spaces. The tissue samples exposed to acute hypobaric hypoxia revealed heightened infiltration of alveolar septae by polymorphonuclear leucocytes (PMN) and interstitial thickening of alveoli with luminal collapse at certain focal points in the descending order of susceptible [Sus(M)] > normal [Nor(M)] > tolerant [Tol(M)] rat lung samples as compared to control [Con(M)] sample (figures 1a-d).

Biochemical parameters : The DCF levels, which are directly proportional to the levels of ROS in the tissues, were found to increase by 37% in Sus(M) and decrease by 71% in Tol(M) samples comparative to levels in Con(M) samples, whereas there was a marginal increase (4.72%) in lung samples of Tol(F) and 35.3% increase in Nor(M) samples as compared to Con(F) and Con(M) samples, respectively (figure 2a). Malondialdehdyde (MDA) assay revealed an

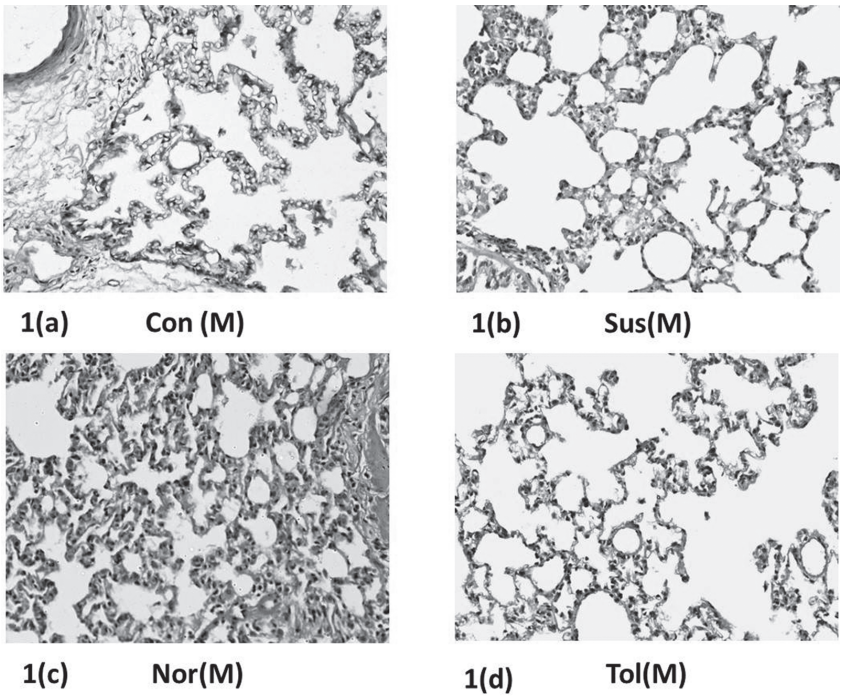

Fig. 1a-d: Histological examination of rat lung tissues of (a) Con(M): control or normoxia male; (b) Sus(M): hypobaric hypoxia susceptible male; (c) Nor(M): hypobaric hypoxia normal male; and (d) Tol(M): hypobaric hypoxia tolerant male.

estimated increase of 55% in peroxide radicals in terms of MDA equivalents in susceptible tissue samples as compared to normoxic or control sample, while only 14-15% increase was recorded in tolerant male and female group samples (figure 2b). The oxidized GSSG content in susceptible tissue was found to be maximum, i.e. 3.56±0.36 μM mg^{-1}, while both tolerant male and female groups exhibited 1.85±0.26 μM mg^{-1} and 1.88±0.29 μM mg^{-1}, respectively and normal samples showing intermediate value of 2.31±0.13 μM mg^{-1} as shown in figure 2c. Superoxide dismutase inhibitory activity decreased in Tol(M) and Tol(F) samples by 42% and 37%, respectively comparative to values recorded in Con(M) and Con(F). An increase in inhibitory activity of SOD by 29.7% in susceptible and 17.6% in normal male samples was recorded as compared to Con(M) rat lung samples (figure 3a). Total catalase activity (42.33%) and total glutathione peroxidase activity (23.37%) observed in Sus(M) samples were the lowest ones compared to the values found in other groups (figures 3b and 3c). Increase of 130%, 89% and 76% in total catalase activity was also recorded in Nor(M), Tol(M) and Tol(F) samples, respectively as compared to Con(M) samples. The glutathione activity in Nor(M), Tol(M) and Tol(F) rat samples remained 115.2%, 144% and 314% (highest), respectively as compared to their normoxic counterparts.

Statistical analysis : One way ANOVA carried out in all the six assays to compare mean values in all four male groups i.e. Con(M), Sus(M), Nor(M) and Tol(M) yielded statistically significant difference ($p < 0.001$) (table 1). Similarly, standard deviation in different groups as revealed by Barlett's statistic (corrected) with p- value > 0.05 in each case led to the conclusion that these samples belonged to the same population, thus, accepting the null hypothesis.

Significant difference of 32.82% ($p < 0.0001$) in mean ROS level values was found when the control group was compared to the tolerant group. On the other hand, difference of 24.82% was estimated between male and female groups ($p < 0.0001$) as shown in table 2. There was no significant difference in control and tolerant group in terms of MDA levels, whereas, 42% total variation of MDA ($p < 0.0005$) was observed in tolerant male and tolerant female.

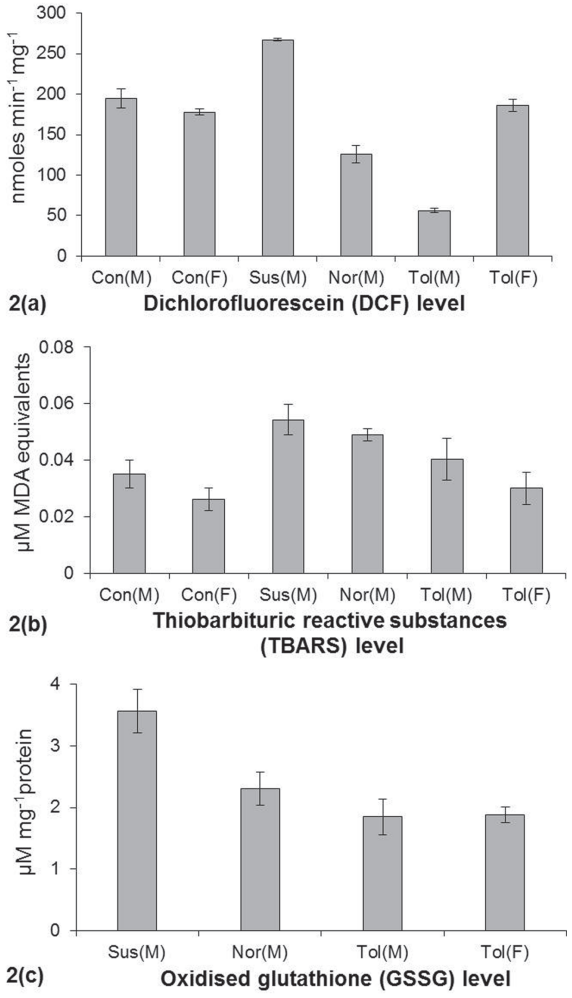

Fig. 2a-c: Estimation of oxidant level in rat lung tissues as determined through (a) dichloro-fuorescein (DCF) directly proportional to reactive oxygen species (ROS), (b) malondial-dehyde (MDA), and (c) oxidized glutathione. Samples studied included Con(M): control or normoxia male; Con(F): control or normoxia female; Sus(M): hypobaric hypoxia susceptible male; Nor(M): hypobaric hypoxia normal male; Tol(M): hypobaric hypoxia tolerant male, and Tol(F): hypobaric hypoxia tolerant female rats.

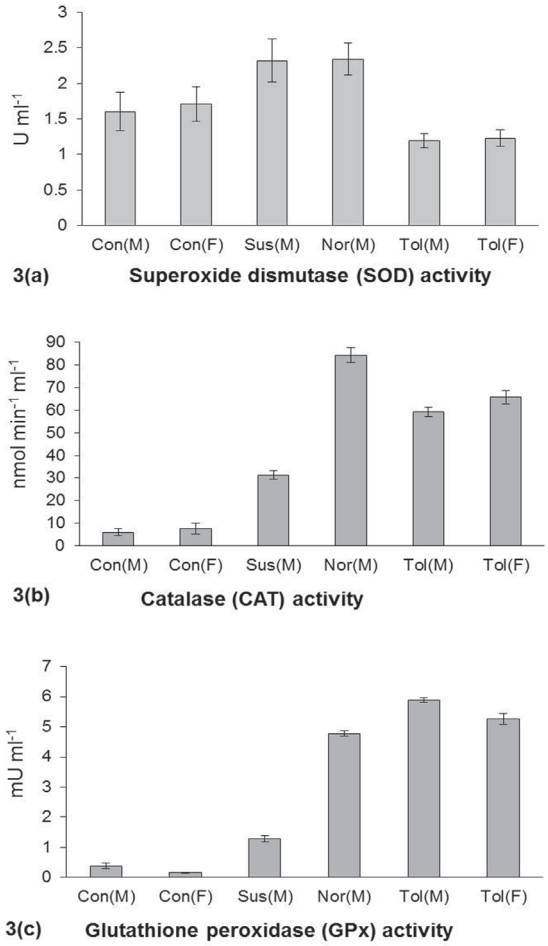

Fig. 3a-c: Antioxidant enzyme activity estimation using different enzyme systems (a) superoxide dismutase, (b) catalase, and (c) glutathione peroxidase in Con(M): control or normoxia male; Con(F): control or normoxia female; Sus(M): hypobaric hypoxia susceptible male ; Nor(M): hypobaric hypoxia normal male; Tol(M): hypobaric hypoxia tolerant male and Tol(F): hypobaric hypoxia tolerant female rats.

Mean SOD activity was 60% significantly different ($p < 0.0001$) in control and tolerant samples, while no significant differences existed in male and female groups. Both tolerant male and female differed with each other ($p < 0.0003$) and with their control counterparts as well in terms of catalase activity ($p < 0.0001$). When unpaired t test (two tailed test) was carried out using average relative values of GSSG assay, experimental sets namely, Tol(F) vs Con (F), and Tol(M) vs Con(M) indicated no significant difference ($t = 0.2377$, degree of freedom =10, $R=0.0056$) as compared by F test statistic ($p < 0.05$). All these results are well corroborated with the graphical representation of mean values shown in figures 2 and 3.

Discussion

During higher altitude ascent, trekkers, army personnel and pilgrims might suffer from acute mountain sickness with symptoms such as shortness of breath, headache, fatigue, nausea, malaise, anorexia, dizziness and mental disturbance. In case of high altitude pulmonary edema, most prominent symptom is the non-productive cough with pink frothy sputum (10). These problems arise due to oxygen depletion in body cells subjected to oxidative stress. Genetic susceptibility of an individual towards development of altitude illness accounts for a significant factor of pathogenesis (11). Hence, extent of oxidative stress in lung tissues of model rats susceptible and tolerant to acute hypobaric hypoxia may differ, when an early phase of hypoxic exposure is examined. The oxidative stress in turn, as stated before, depends upon the balance between oxidants and antioxidants. Therefore, we studied various biochemical parameters and histological changes, which could describe the pathophysiological state of the tissue.

Table 1 Results of statistical analysis performed in all four male rat groups including Con(M), Sus(M), Nor(M) and Tol(M) using one way ANOVA, F test and Barlett's statistic.

Parameter	ROS level	MDA equivalent	GSSG level	SOD activity	CAT activity	GPX activity
Number of samples	24	24	18	24	24	24
R^2	0.997	0.702	0.871	0.836	0.995	0.998
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Statistically significant difference in means($p < 0.05$)	Yes	Yes	Yes	Yes	Yes	Yes
F (Dfn, Dfd)*	F (3, 20) = 2647	F (3, 20) = 15.71	F (2, 15) = 50.46	F (3, 20) = 34.03	F (3, 20) = 1296	F (3, 20) = 4730
Bartlett's statistic (corrected)	7.829	5.636	0.4101	4.800	2.992	0.5034
p-value	0.052	0.1307	0.8146	0.1871	0.3928	0.9182
Statistically significant difference in standard deviation ($p < 0.05$)	No	No	No	No	No	No

*Dfn: Degree of Freedom numerator; Dfd: Degree of freedom denominator

Table 2 Two way ANOVA with F test statistic to compare oxidant and antioxidant levels in hypobaric hypoxia tolerant male i.e. Tol(M) and female Tol(F) rats with each other and with control male and female samples, respectively.

Assay	Variation	Interaction	Control vs Tolerant	Tol(M) vs Tol (F)
ROS levels	% of total variation	41.86	32.82	24.84
	p-value	<0.0001*	<0.0001*	<0.0001*
	F (Dfn, Dfd)**	F (1, 20) = 1740	F (1, 20) = 1364	F (1, 20) = 1033
MDA equivalent levels	% of total variation	0.1639	9.635	41.96
	p-value	0.7970	0.0594	0.0005*
	F (Dfn, Dfd)	F (1, 20) = 0.06797	F (1, 20) = 3.995	F (1, 20) = 17.40
SOD activity	% of total variation	0.3242	59.59	1.555
	p-value	0.6860	< 0.0001*	0.3797
	F (Dfn, Dfd)	F (1, 20) = 0.1683	F (1, 20) = 30.93	F (1, 20) = 0.8070
CAT activity	% of total variation	0.1872	98.73	0.5291
	p-value	0.0171	< 0.0001*	0.0003*
	F (Dfn, Dfd)	F (1, 20) = 6.764	F (1, 20) = 3567	F (1, 20) = 19.11
GPx activity	% of total variation	0.1443	99.10	0.6258
	p-value	0.0001*	<0.0001*	< 0.0001*
	F (Dfn, Dfd)	F (1, 20) = 21.81	F (1, 20) = 14980	F (1, 20) = 94.59

*Statistically significant variation as p-value was found to be < 0.05.
** Dfn: Degree of Freedom numerator; Dfd: Degree of freedom denominator.

The preliminary histological observations highlighted the damaged alveolar interstitia and profound neutrophil infiltration in injured susceptible lung tissues as compared to normal and tolerant lung samples (figures 1a-d). The alveolar macrophages were almost depleted in susceptible and normal samples as compared to control and tolerant tissue samples, which along with interstitial edema might have caused increased infiltration neutrophils as occurs during acute lung injury (5). As otherwise, the lung alveolar macrophages secrete anti-inflammatory molecules and regulate this infiltration (12).

A few earlier studies have reported that antioxidant protein expression and activity in blood increases in hypobaric hypoxia tolerant rats compared to susceptible rats (13,14,15). We evaluated changes in the oxidant levels and antioxidant activity in lung tissues during an early phase of acute hypobaric hypoxia in order to determine the differential response of the susceptible and tolerant rats to the oxidative stress. Markedly increased ROS levels in Sus(M) samples signal a greater necessity to decrease ROS levels by increasing activity of antioxidant machinery as evident from highest SOD levels in these susceptible rats. However, reduction in catalase and glutathione peroxidase activity indicates inefficient removal of ROS from the cellular systems, as against effective treatment and clearance of ROS as observed through

increased levels of activities found in both Tol(M) and Tol(F) rats. Marginal increase in the level of reactive oxygen species in tolerant female rat samples may enhance the life span during acute hypobaric hypoxia as reported in some earlier studies, which proved that increased longevity and tissue regeneration results from increased ROS levels and ROS mediated signaling (16,17). It was long ago observed that MDA levels, SOD and Gpx activity rise in rat lungs during exposure to hypobaric hypoxia (18). In our study, we found the highest increase in oxidant MDA, ROS and GSSG in lungs of susceptible rats as compared to other groups, as expected from their physiological susceptibility to acute hypobaric hypoxia. Higher SOD activity in susceptible rat lungs also indicates higher production of hydrogen peroxide, which ultimately contributes to ROS levels, signaling and lipid peroxidation. Other antioxidant enzymes such as CAT and Gpx were observed to show maximum activity in normal male, tolerant male and female samples. Our results corroborate well with our previous study on antioxidant enzyme activity in blood plasma, when same groups of rats were exposed to identical simulated altitude conditions (6). However, in our tissue-specific study, we also studied normoxic male and female along with tolerant females to figure out gender-specific differences.

These parameters studied in the present study suggest that the susceptible, normal and tolerant rats exhibit physiological differences even in the early phase response to acute hypobaric hypoxia. Thus, these physiologically different groups can further be studied in terms of global gene expression and proteome studies to explore the further cause of such variability as well as gender biasness towards development of higher altitude illness. This will also lead to the development of specific markers in terms of proteins and genes, as oxidative parameters though showed difference, but cannot be solely used to distinctly diagnose physiologically different groups.

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Declaration of Interest *:* The authors report no conflicts of interest and are alone responsible for the content and writing of the paper.

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Molecular Identity Using Inter-Simple Sequence Repeat & Random Amplified Polymorphic DNA Markers in Soybean (*Glycine Max*) Cultivars

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Abstract

Plant molecular identity (ID) is necessary to describe molecular characteristics of plants, which should contain all of the required information. Using Inter-Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) primers, molecular ID can be described. Complete molecular ID system is described in this study for Soybean, which is important for the modern breeding and biotechnology point of view. Using five Soybean cultivars, we analysed the products of PCR with ISSR and RAPD primers and discussed the strategy for establishing their molecular ID. Using the segmented naming method, we designate the simple names and the full name systems of five Soybean cultivars.

Key words: Soybean; Molecular identity; Simple name; Full name; segmented naming method

Introduction

DNA markers have proven to be an efficient tool for molecular characterization in plant breeding, and are widely used in fingerprinting, diversity analyses, and gene mapping (1; 2). Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers, on the other hand, require only small amount of DNA sample without involving radioactivity tests and are simpler as well as faster. RAPD has proven to be quite efficient in detecting genetic variations (3), even in closely related organisms such as two near isogenic lines (NIL). ISSR technique is also very simple, fast, cost-effective, highly discriminative and reliable (4). At present, RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micro-propagated material in various plants (5; 6; 7; 8).

The inter simple sequence repeat (ISSR) technique is another PCR-based method, which involves amplification of the DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite directions. The technique uses microsatellites, usually 16- 25-bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to mainly amplify ISSR sequences of different sizes. ISSR-PCR is a simple, quick, and efficient technique with high reproducibility. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology (9; 1). Random amplified polymorphic DNA (RAPD) is easy to perform and requires no information about the DNA sequence to be amplified. It has been used, therefore, to study the genetic diversity in various plant species (10; 11), authenticate the herbal medicinal materials (12), and detect adulterants (13; 14). These molecular IDs have proven very helpful in spice classification, identification, and germplasm protection. However, molecular IDs can vary a lot depending on the methods for naming the IDs as well as the construction system of the molecular IDs. It would be possible to establish

a plant ISSR and RAPD based ID system if a standard naming system were developed that contains enough information to reflect PCR conditions, such as primers used in the experiment for a certain plant, annealing temperatures, and PCR products. In general, the same crop should have the same molecular ID, and different plants should be distinguished with ISSR and RAPD markers. Using five Soybean cultivars, a complete ISSR and RAPD based molecular ID system is described in this study, which can be easily used and expanded with much more information. The objective of this study is an attempt to develop a plant molecular identity system in soybean using the DNA banding patterns from RAPD and ISSR marker systems. This can be useful in identity testing of plant varieties especially in case of disputes with respect to varietal identification.

Material and Methods

Five commonly used major Soybean (*Glycine Max*) cultivars ASO-138, ASO-104, ASO-NRC37, ASO-JS9305 and ASO-JS335 were taken in this study (Table 1 and Fig. 1). The all five cultivar showing different growth pattern in terms of Flower color, Pods hair, Days to 50% flowering and Plant height (cm.) and on the basis of above characters these cultivar were finalized. After election Genomic DNA was isolated by a modified cetyl-trimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990). Genomic DNA was quantified on 0.8% and later NanoDrop Spectrophotometer. PCR was performed with 8 RAPD and 5 ISSR markers (Table 2). The PCR mixture contained: approximately 50 ng template DNA, 10 µL 2X Taq MasterMix, 1 µL 10 µM primer, and topped with distilled water to a total volume of 20 µL. The PCR program was run as follows: 5 min at 95°C for pre-denaturation; 36 cycles each at 94°C for 1 min, step down annealing temperature (Tm in°C,) for 1 min, and 72°C for 1 min; with a final extension at 72°C for 10 min. PCR products were electrophoresed on 1.8% agarose gels. DNA fragment size (bp) was calculated with gel documentation system (Bio-Rad) by comparing the DNA bands to a DNA ladder (Merck).

Results

Proper ISSR and RAPD primers were selected based on clearly detectable PCR bands under optimized PCR conditions. The reaction products were electrophoresed on a 1.8% agarose gel. Clearly detectable amplified ISSR and RAPD bands were obtained ranging in size from 120 to 1200 bp for the five Soybean cultivars. Among the 14 primers, 5 were suitable ASO-138, ASO-104, ASO-NRC37, ASO-JS9305 and ASO-JS335. Out of five primers three RAPD (RIPC3, RPIC4 and OPB4) and two ISSR (B17899 and HB12) could be used for all five cultivars, and there were no PCR products observed for eight primers (RPIC 7, OPY 11, RPIC 9, OPB 4, B17898, A17898, HB10, and OPG 4).

The ISSR and RAPD fingerprinting profile for ASO-138, ASO-104, ASO-NRC37, ASO-JS9305 and ASO-JS335, based on the selected primers, are shown in Figure 2. A complete molecular ID was used to describe the ISSR and

Table 2. List of markers used in this study

Sr. No.	Primers	Tm (in °C)
1	RPIC 3 (RAPD)	35
2	RPIC 7(RAPD)	35
3	OPY 11(RAPD)	32.8
4	OPB 4(RAPD)	26.4
5	RPIC 9(RAPD)	35
6	OPB 4(RAPD)	26.4
7	B17899(ISSR)	47.7
8	B17898(ISSR)	44.7
9	A17898(ISSR)	40.1
10	HB10(ISSR)	37.4
11	HB12(ISSR)	56.2
12	RM1 (R,F)(SSR)	66.3
13	OPG 4(RAPD)	29.2
14	RPIC 4(RAPD)	35

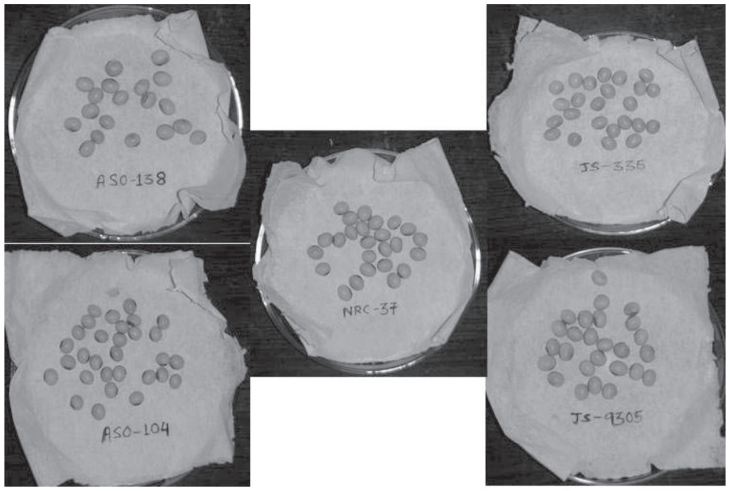

Fig.1. Seed Morphology of Five Soybean Varieties

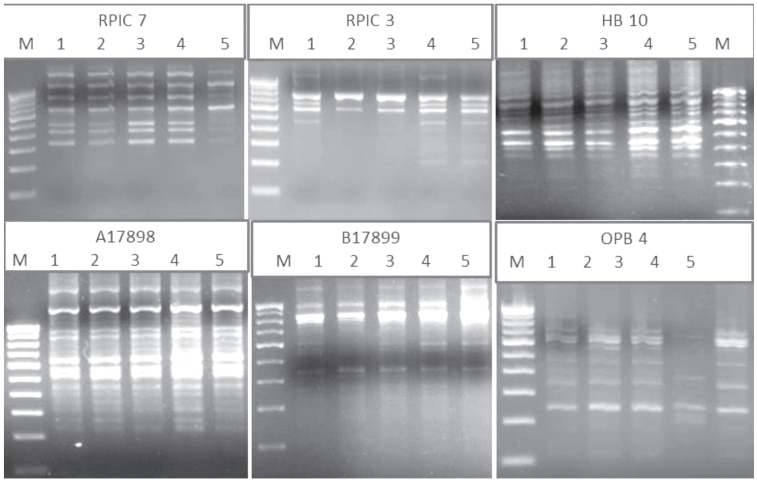

Fig. 2. ISSR and RAPD based fingerprinting profile of five Soybean cultivars with different primers. (M: 100bp DNA ladder and 1 to 5: AS0-138, AS0-104, AS0-NRC37, AS0-JS9305 and AS0-JS335)

Table 1. List of five genotypes used in this study and their morphological traits

Seed type Features	**AS0-138 (1)**	**AS0-104 (2)**	**AS0-NRC37 (3)**	**AS0-JS9305 (4)**	**AS0-JS335 (5)**
Flower color	PINK	WHITE	WHITE	PINK	PINK
Pods hair	SMOOTH	HAIRY	HAIRY	SMOOTH	SMOOTH
Days to 50% flowering	35	35	46	41	40
Plant height (cm.)	60	80	50	40	50

RAPD fingerprinting features for a certain plant. The ID should contain the molecular ID naming part and the related explanatory part. The explanatory part should contain information related to the suitable primer sequences, optimized annealing temperature for the ISSR and RAPD-based PCR reaction, the PCR reaction system, and the electrophoresis image. Considering the informative characteristics and ease of use, we named the molecular ID with two forms: a simple name and a full-informative name. For the simple name, we designated the molecular ID with numbers reflecting the bands obtained from PCR. The complete name contains detailed information of the ISSR fingerprint profile

The simple name was designed with six digital serial numbers (Figure 3). The 1st five letters (for RAPD primers and six latter in case of ISSR markers) digits represent the primer name (e.g., RPIC), and the 4th represents band numbers smaller than and including 500 bp, the 5th represents band numbers between 500 and 1000 bp, and the 6th represents band numbers larger than 1000 bp.

The full-informative name should contain all of the details of the ISSR fingerprinting profile. It is segregated into four parts, which include name of primer (A), total no of bands (B), PCR bands number (C), I, II, III, etc., to indicate the PCR fragment size (bp), and a final part, @ablr which provides information about the institute (Figure 4).

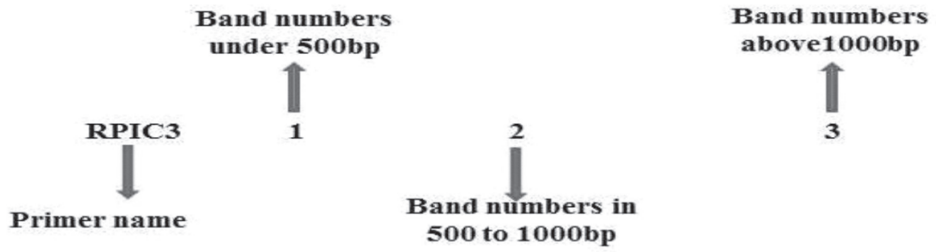

Fig. 3. Demonstration of the simple name

Fig. 3. Demonstration of the simple name

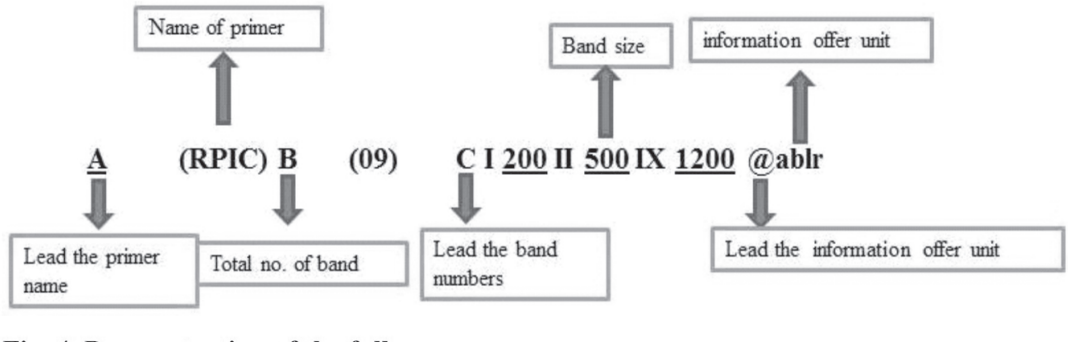

Fig. 4. Demonstration of the full name

Simple name of ASO-138
RPIC3241-RPIC4021-B17899431-HB12440-OPB4730
Simple name of ASO-NRC37
OPB4730-RPIC3030-RPIC4022-B17899441-HB12430
Simple name of ASO-104
RPIC3030-RPIC4021-B17899241-HB12430-OPB4730
Simple name of ASO-JS9305
RPIC3331-RPIC4021-B17899441-HB12330-OPB510
Simple nam
RPIC3331-RPIC4031-B17899441-HB12330-OPB4740

Full name of ASO-138
ARPIC3B07CI200II450III600IV700V800VI1000VII1200@ablr
ARIPC-4B03CI800II1000III1150@ablr
AB17899B09CI280II350III450V500VI600VII700VII1000IX1200@ablr
AHB12B08CI380II400III420IV490V550VI590VII700VIII800@ablr
AOPB4B10CI120II180III250IV290V400VI450VII500VIII600IX700X900@ablr
Full name of ASO-NRC37
AOPB4B10CI120II180III250IV290V400VI450VII498VIII600IX700X900@ablr
ARPIC3B03CI600II700III750@ablr
ARPIC4B04CI750II900III1100IV1200@ablr
AB17899B10CI280II350III450IV500V600VI700VIII750IX1000X1200@ablr
AHB12B07CI380II400III420IV490V550VI700VII800@ablr
Full name of ASO-104
ARPIC3B03CI600II700III750@ablr
ARPIC4B03CI850II1000III1200@ablr
AB17899B07CI280II350III600IV700V750VI1000VII1200@ablr
AHB12B07CI380II400III420IV490V550VI700VII800@ablr
AOPB4B10CI120II180III250IV290V400VI450VII498VIII600IX604X700@ablr
Full name of ASO-JS9305
ARPIC3B07CI200II250III450IV600V700VI800VII1100@ablr
ARPIC4B03CI750II1000III1200@ablr
AB17899B09CI280II350III450IV500V600VI700VII750VIII1000IX1200@ablr
ABH12B06CI350II400III420IV550V700VI800@ablr
AOPB4B06CI200II220III250IV460V498VI700@ablr
Full name of ASO-JS335
ARPIC3B07CI200II250III450IV600V700VI800VII1100@ablr
ARPIC4B04CI700II750II1000IV1200@ablr
AB17899B09CI280II250III450IV500V600VI700VII750VIII1000IX1200@ablr
AHB12B06CI380II400III420IV550V700VI800@ablr
AOPB4B11CI120II180III280IV290V400VI450VII498VIII600IX604X700XI900@ablr

Discussion

ISSR marker analysis is an easy, fast, inexpensive, accurate, and reliable technique for genetic analyses of various plant species (2). A complete molecular ID should contain all of the detailed information of target plants. To establish the representative molecular ID, it is critical to collect and reflect all of the information from the amplified ISSR profile. The molecular IDs currently used are relatively quite simple and

incomplete. Most of the time, the PCR band is scored as 1 when it is present and as 0 when it absent, and serial numbers are assigned to molecular IDs based on PCR results (16; 17; 18; 19). In 2009, a molecular identity (ID) dataset, based on numeralized data from polyacrylamide gel electrophoresis (PAGE) bands, was established with ID Analysis software for 83 Soybean cutivars in Heilongjiang, China (20). Subsequently, more molecular IDs of more plants were established in China, including those of sweet sorghum (21) kenaf (22), flax (23), hybrid rice (19), peach (24), sugarcane (25), peanut (17), and Spiraea (16). Such molecular IDs are difficult to be widely used across plants due to their limitations and low information content. In the present study, we established naming systems, which fully reflect the plant characteristics of molecular ID. The names contain information related to primers, the PCR reaction system, bands obtained, and the information provider. The system is also easy to use. For example, primer RPIC3 can be used to distinguish ASO-138 and ASO-NRC37, ASO-JS9305 with their simple names RPIC3241, RPIC3030 and RPIC3331, it is easy to find the full name difference between ASO-138 (ARPIC3B07CI200II45 0III600IV700V8 00VI11000VII1200@ablr), ASO-NRC37 (ARPIC 3B 03C I60 0II700III750@ablr) and ASO-JS9305 (ARPIC3B07C I200II250III4 50IV600V700VI8 00VII1100@ablr). Both simple and full names can be easily used or combined according to specific needs. As the 1st four letters represent the primer name, the molecular ID database can also be easily expanded when more primers are introduced into the experiment. For this system, more than 999 primers can be chosen for one plant.

In conclusion, we designated a complete naming system that includes descriptive information and molecular ID information. The descriptive part should contain the detailed information of ISSR and RAPD amplification conditions, and the molecular ID part should contain the simple name and full name system using the segmented naming method. Therefore, a new complete molecular ID system was established, which can be easily used and expanded as more information becomes available. Hopefully, this system will provide an improved solution for the characterization of ISSR and RAPD markers.

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Somatic Embryogenesis of *Caralluma sarkariae* Lavranos & Frandsen- An Important Medicinal Plant

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Abstract

An efficient procedure has been developed for inducing somatic embryogenesis from mature internodes of *Caralluma sarkariae* an important medicinal plant. Internodal explants cultured on Murashige and Skoog's (MS) medium supplemented with picloram 2.0 mg/l was very effective in inducing somatic embryos. Globular stage embryos developed on induction medium containing picloram 2.0 mg/l. These embryos developed into torpedo and cotyledon stage embryos upon sub culture on medium containing MS salts + B_5 vitamins + BAP 2.0 mg/l + 2-iP 1.0 mg/l + NAA 0.5 mg/l with 3% sucrose. About 100 mg of callus induce more than 100 embryos. Embryo germination and plantlet formation was achieved by sub culturing mature embryos on fresh sub culturing medium (MS salts + B_5 vitamins + BAP 2.0 mg/l + 2-iP 1.0 mg/l + NAA 0.5 mg/l). The plantlets were acclimatized under field conditions with 75% survival. This simple regeneration system through somatic embryogenesis may be beneficial for mass propagation of *Caralluma sarkariae*.

Key words: *Caralluma sarkariae*; endemic, medicinal plant, somatic embryogenesis.

Introduction

In vitro clonal propagation of medicinal plants enables large - scale production of therapeutically high value taxa for commercialization and sustainable utilization in the industrial sector. *Caralluma sarkariae*, a member of Asclepiadaceae, is an important indigenous medicinal plant with restricted distribution in India. *Caralluma sarkariae* is an endemic succulent herb, growing in Nagamalai hills, in Madurai district in Tamilnadu. Plants belonging to this genus are rich in esterified polyhydroxypregnane glycosides, some of which showed antitumour activity and others were postulated as precursors of cardenolides (1, 2). The genus is also characterized by the presence of flavone glycosides (3,4). The hypoglycemic effect of aqueous and alcoholic extracts of whole plant of C*aralluma* species was investigated by Jayakar *et al.* (5) in both normal and alloxan induced diabetic rats. The species of *Caralluma* found in India are edible and the medicinal properties of *Caralluma* include anti-inflammatory, anti-nociceptive, anti-ulcer, antidiabetic, carminative, febrifungal, anti-pyretic and antioxident effects. *Caralluma* extracts have also been found to be appetite suppressant, a property which is well known to Indian tribal and hunters. Indian folklore records its use as a potent appetite suppressant and weight loss promoter. Some *Caralluma* species are used in the treatment of obesity. The extract of *Caralluma* sp. in the form of capsules has been released under trade name GENASLIM for body weight control (6).

Apparently due to non – availability of sufficient quality planting materials, commercial plantations of this important medicinal species have not been widely attempted and presently only the wild population is exploited for extraction purposes. Due to overexploitation and lack of

organized cultivation, the wild population is declining fast. Overexploitation of natural stands has caused depletion of these plants in nature. There are a number of constraints for the propagation and conservation through conventional methods like vegetative and seed propagation.

In our present study propagation of *Caralluma sarkariae* through seed is held back by low percentage of viability and low germination rate of seeds. The major ones are variations in edaphic and climatic factors, low percentage of seed set and seasonal dormancy. The propagation in its natural habitat is a rare phenomenon evidenced by close field observation. The above mentioned causes prompted us to find an alternate method of rapid propagation of this species.

In view of medicinal importance there is an urgent need to conserve this species *ex - situ* through *in vitro* methods. Sreelatha *et al.* (2008, 2009) reported micropropagation of *Caralluma sarkariae* through axillary buds but there are no reports on somatic embryogenesis in this species. The aim of this work is to present the *in vitro* propagation of *Caralluma sarkariae* through somatic embryogenesis.

Materials and Methods

Caralluma sarkariae was collected from Nagamalai hills of Madurai district in Tamilnadu. The stems were potted in pots and maintained at Botanical garden, Botany department, Sri Krishnadevaraya University, Anantapur. Plants of *Caralluma sarkariae* with actively growing shoots were collected from the garden to provide the explant source. The shoots were separated from the plant defoliated and cut into small pieces of about 5-8 cm length. The shoot pieces were initially disinfected by rinsing in 1% (v/v) Tween – 20 (Merck) for five minutes with constant agitation. Then they were rinsed in distilled water thrice and then taken into the laminar air flow chamber where they were rinsed with sterile double distilled water. The explant material was immersed in 70% ethanol for 1 min. followed by surface sterilization in aqueous solution of 0.1% (w/v) $HgCl_2$ for five min. and 3-4 rinses in sterilized double distilled water. Stem segments 0.5-1.0 cm long containing the internodal region was aseptically cut from the surface sterilized shoot pieces and inoculated on to the sterilized nutrient medium one explant /tube.

The basal medium used in the present study was MS (9) medium and MS salts + B_5 vitamins (10) medium. Addition of plant growth regulators to medium was done with filter sterilization in aseptic conditions and unless it is specified all media were fortified with 3% sucrose (w/v) and 0.8% agar. The p^H of medium was adjusted to 5.8 prior to the addition of agar and autoclaved at 121ºC for 15 min. All cultures were incubated at 25 ± 2ºC and under 16h light / 8h dark photoperiod with light intensity of 50ìE m^{-2} s^{-2} provided by cool – white fluorescent lamps.

Internodal explants were inoculated on MS medium containing 2,4-D, Picloram, 2,4,5-T and 2,4,5-TP. Embryogenic callus with globular stage embryos were transferred onto following embryo maturation media: (a) medium containing MS medium (EM0) + 3% sucrose, solidified with 0.8% agar; (b) medium containing MS medium + (BAP 2.0 mg/l (EM1), BAP 2.0 mg/l + NAA 0.5 mg/l (EM2) and BAP 2.0 mg/l + 2-ip 1.0 mg/l + NAA 0.5 mg/l (EM3)) + 3% sucrose, solidified with 0.8% agar; (c) medium containing MS salts + B_5 vitamins (10) + 3% sucrose (EM4) solidified with 0.8% agar; (d) medium containing MS salts + B_5 vitamins + BAP 2.0 mg/l + NAA 0.5 mg/l (EM5), 2-iP + NAA 0.5 mg/l (EM6), BAP 2.0 mg/l + 2-iP 1.0 mg/l + NAA 0.5 mg/l (EM7)) + 3% sucrose, solidified with 0.8% agar. All the experiments were repeated thrice and twenty explants were used per treatment. Then 3.0 to 4.0 mm long somatic embryos (cotyledonary stage) were isolated from embryogenic cultures and transferred to EM4 medium then after 20 days they were transferred to EM7 medium for germination.

In vitro developed plantlets were taken out of culture tubes, washed under running tap water

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to remove agar, and transplanted into plastic pots containing vermiculate. The plants were covered with polythene bags and kept in a culture room at 25 ± 2°C temperature and 16h/day illumination with cool fluorescent light for 15 days. The plantlets were every day irrigated with ½ strength MS solution without sucrose. After 2 weeks, the plants individually transferred to pots containing soil, sand and farmyard manure (1:1:1) and were reared in the greenhouse and subsequently to field conditions.

For histological studies, the calli with somatic embryos were fixed for 24h in FAA (formalin + glacial acetic acid + 70% ethanol). Following dehydration tertiary butanol the material was in filtered and embedded in paraffin wax. The paraffin blocks were cut using a Spencer microtome (USA) attached with a steel knife. The sections were mounted on microslides dewaxed and stained with safranin. Photographs were taken under Nikon light microscope.

Results and Discussion

Mature internodal segments cultured on MS medium supplemented with various auxins like 2,4-D, Picloram, 2,4,5-T and 2,4,5,-TP expanded in size during the 15 days of culture and developed callus at cut ends. In another 2 weeks, entire explants were covered with callus masses but texture, amount and colour varied depending on the type and concentration of the growth regulators. The explants cultured on growth regulator free medium did not respond and became necrotic. Embryogenic callus formed from explants cultured on media that contained various hormones are shown in Table – 1.

Non embryogenic calli were induced with the internodal segments in most of media containing different concentrations of 2,4-D (1.0 mg/l to 3.0 mg/l), picloram (1.0 mg/l and 3.0 mg/l), 2.4.5-T (0.5 mg/l and 1.0 mg/l) and 2.4,5-TP (1.0 mg/l and 2.0 mg/l). The quantity and quality

Table 1. Effect of different auxins on somatic embryogenesis from internodal explants of *Caralluma sarkariae*

Plant growth regulators	Concentration mg/l	Percent of cultures showing response	Number of embryos / explant	callusing
2,4-D	1.0	30	-	++
	2.0	85	-	+++
	3.0	70	-	+++
picloram	1.0	50	-	+++
	2.0	70	35	++
	3.0	55	-	+++
2,4,5-T	0.1	50	20	++
	0.5	55	-	+++
	1.0	75	-	++++
2,4,5-TP	0.5	46	25	+
	1.0	70	-	+++
	2.0	65	-	+++

of callus formed was more in 2.4-D when compared to other concentrations. Embryogenic calli formed from explants cultured on media that contained picloram (2.0 mg/l), 2.4,5-T (0.1 mg/l) and 2,4,5-TP (0.5 mg/l). The concentration of picloram (2.0 mg/l) in medium significantly affected the number of embryos production compared to other concentrations (Table 1). The calli were green, granular and nodular and they were bearing globular stage embryos on the surface particularly 2.0 mg/l of picloram alone resulted in an average of 35 globular embryos (Table 1) (Fig 1A).

Somatic embryo development stopped at the globular stage. A maturation treatment was necessary for the further development of *Caralluma sarkariae* somatic embryos. Embryogenic calli of *Caralluma sarkariae* were cultured on MS medium (EM0) supplemented with different levels of cytokinins and auxin concentrations (EM1, EM2 and EM3) and MS salts and B_5 vitamins medium (EM4) containing (8) various levels of cytokinins and auxin concentrations (EM5, EM6 and EM7). Different levels of MS medium EM0, EM1, EM2 and EM3 and MS salts and B_5 vitamins medium EM4, EM5 and EM6 did not induce maturation. However, the embryogenic calli cultured on EM7 medium produced torpedo and cotyledon stage embryos with 20 days of culture (Fig 1B). Similar beneficial effect of cytokinins (alone or combination with auxins) on somatic embryogenesis has been demonstrated in *Gymnema sylvestre* (11), and in rose (12, 13).

Mature somatic embryos (cotyledon stage) were sub-cultured on EM8 medium for germination. Sixty percent of somatic embryos germinated into plantlets in 5 weeks (Fig 1C). The regenerated plants were transplanted to small plastic pots containing vermiculate and kept in culture room for 10 days. The plants were irrigated once in 2 days with ½ strength MS solution. Then plants were individually transferred to pots containing soil, sand and farmyard manure (1:1:1) and were reared in the greenhouse. The plantlets were acclimatized

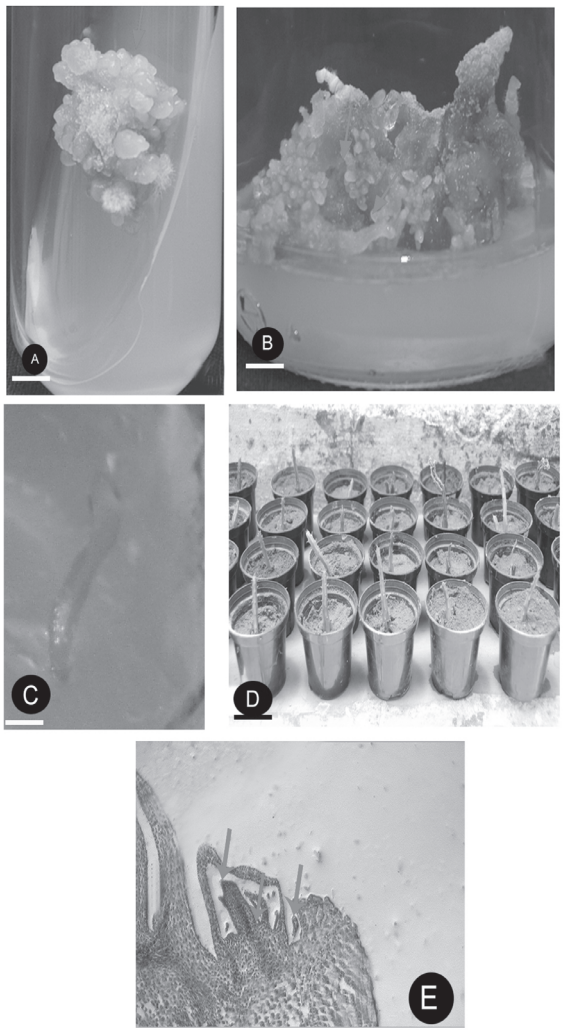

Fig.1. Somatic Embryogenesis of *Caralluma sarkariae*

A. Globular stage somatic embryos developed on embryogenic callus on medium containing MS+ Picloram 2.0 mg/l.B. Torpedo and cotyledon stage embryos developed on MS salts + B_5vitamins + BAP 2.0 mg/l + 2-iP 1.0 mg/l + NAA 0.5 mg/l. C. Germination of somatic embryos. D. Plants obtained from somatic embryos transferred to soil (after 20 days). E. A section of a 35 d-old callus showing initiation of heart shaped and cotyledonary shape stages and showing initiation of shoots from the meristems (arrow marks X 100).

under field conditions with 75% survivability rate (Fig 1D).

The histological analysis of the somatic embryo producing regions confirmed that the induction of the development process was embryogenic nature. Development of somatic embryos appeared to progress through typical globular, heart, torpedo and cotyledonary stage embryo development. Light microscopic observations of embryogenic mass revealed the presence of nodular structures containing cytoplasmic cells at the central region. The heart shape stage embryo (Fig 1E) was bilaterally symmetrical. The cotyledonary stage embryos showed the presence of two prominent cotyledons (Fig 1E). Some of the structures also has vascular tissue with bipolar meristems which ultimately surrounded by parenchymatous tissue. At this stage, development of clear bipolar embryos with organized shoots portion was observed (Fig 1E).

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Biological Properties of Xylooligosaccharides as an Emerging Prebiotic and Future Perspective

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Abstract

Metabolism of complex carbohydrates by probiotics in human gastrointestinal (GI) tract is generally accepted to significantly improve the health and resistance to diseases for the host. Presence of sufficient amounts of carbohydrates that selectively can stimulate the growth and viability of these beneficial bacteria is imperative. Some probiotic species of bifidobacteria and lactobacilli have the ability to utilize xylooligosaccharides (XOS) and produce short chain fatty acids which is having beneficial effect on health. Current article reviews the results of several studies available on degradation and utilization of XOS by beneficial gut microbiota and discusses their future prospective as emergent prebiotic in synbiotic preparations with reference to biological effects observed on human and animal health.

Key words: xylooligosaccharides, prebiotic, *Weissella,* xylanase, immunomodulatory, gut microflora

Introduction

Diet is an important determinant of disease risk associated with changing lifestyle in population of all age groups. Recent evidences support that functional food ingredients can have an impact on a number of gut-related diseases. In colon, probiotic bacteria and the saccharolytic microbiota utilize the carbohydrates that resist hydrolysis by human digestive enzymes and are not absorbed on transit through the small intestine. Non-digestible oligosaccharides (NDOs) which beneficially affect the host by selectively stimulating the growth and D or activity of one or a limited number of probiotic bacteria in the colon are termed as prebiotics (1, 2). More refined definition of prebiotic states that a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits Gibson (3). To date, probiotics and prebiotics are predominantly used in food, and their application in medicine is rising significantly. Consumption of probiotic in combination with a suitable prebiotic (synbiotic) can result in synergistic effects, by improving the growth of the strain in the colon and by increasing autochthonous bacteria. Prebiotics possess remarkable functional and physiological attributes such as low energetic value, low sweetness, non-cariogenicity, and hypolipidaemic and hypocholesterolaemic properties (4). They are indigestible by human gastrointestinal enzymes and are not degraded by low gastric pH; however, they are fermented in the large bowel and enhance the population of beneficial microbes such as lactobacilli and bifidobacteria (5). Postulated health benefits of prebiotics include immunomodulation, inhibition of pathogens, stimulation of calcium absorption and effectiveness in colon cancer.

Some well known prebiotics are inulin, lactulose, fructooligosaccharide (FOS),

galactooligosaccharide (GOS), isomalto oligosaccharides and lactosucrose. Numerous researchers have demonstrated potential prebiotic effects of GOS and FOS on the growth of bifidobacteria and lactobacilli (6, 7, 8). Currently, xylooligosaccharides (XOS) is also an emerging prebiotic that are sugar oligomers made up of xylose units and obtained from bamboo shoots, fruits, vegetables, milk, and honey (9, 10).

Depending upon various xylan sources used for XOS production, the structures of XOS vary in degree of polymerization (DP), monomeric units, and types of linkages. Generally, XOS are mixtures of oligosaccharides formed by xylose residues linked through β-(1→4)-linkages (2, 4). The number of xylose residues involved in their formation can vary from 2 to 10 and they are known as xylobiose, xylotriose, and so on. XOS also exhibits a various bioactive properties such as reducing cholesterol, anti-microbial, anti-inflammatory, anti-allergy and improves mineral absorption (4, 11). In addition, XOS show favourable technological features including stability in wide range of pH, resistance to heat, ability to offer lower available energy and ability to achieve significant biological effects at low daily intakes which would be useful for food industry (9). Bifidobacteria possess a wide range of genes involved in carbohydrate catabolism (12) and have been described for their ability to grow on FOS and XOS (13, 14). Prebiotic effect of XOS has not yet been effectively exploited for LAB compared to FOS and GOS (15, 16).

Production and purification of XOS : XOS are obtained from xylan containing lignocellulosic materials (LCMs) by chemical methods, direct enzymatic hydrolysis of a susceptible substrate or a combination of chemical and enzymatic treatments (17, 18). The production of XOS with chemical methods can be accomplished by steam, diluted solutions of mineral acids, or alkaline solutions. However, extraction of xylan with steam or acid produces large amounts of monosaccharides and their dehydration products (18, 19).

The usual purity of commercial XOS lies in the range of 75% to 95%. Thus, to produce food-grade XOS, the autohydrolysis liquors have to be refined by removing both monosaccharides and nonsaccharide compounds to obtain a concentrate with an XOS content as high as possible (4). Solvent extraction and precipitation, adsorption and chromatographic separation are most commonly employed methods for the purification of XOS. Though expensive, ultrafiltration and nanofiltration are the most promising methods for refining and concentrating oligosaccharides.

Degradation and utilization of XOS by bacteria : The degradation and utilization of XOS are strain-specific and are also affected by the DP of oligomers present in XOS mixture (4). *Bifidobacterium* strains are found to efficiently ferment XOS and to produce metabolically active compounds such as short chain fatty acids (SCFA) (20, 21). Only some *Lactobacillus* strains have been found to utilize XOS. In a study conducted by Crittenden et al. (2), they reported that except *L. brevis* none of the tested *Lactobacilli* showed XOS utilization during fermentation. However, in another study Van Laere et al. (22) found that an *L. acidophilus* strain was able to ferment xylobiose, -triose, and -tetraose as revealed by high Performance Anion Exchange Chromatography (HPAEC) analysis of the samples.

Madhukumar and Muralikrishna (23) reported that XOSs from wheat bran are having more prebiotic activity than Bengal gram husk xylooligosaccharides as indicated by their prebiotic activity experiments, which may be due to their relatively higher arabinose content. Out of all the microorganisms tested *P. pentosaceus* NCDO 813 and *L. brevis* NDRI strain RTS utilized more effectively both WBO and BGO followed by *P. pentosaceus* ATCC 8081, *B. adolescentis* NDRI 236, *B. bifidum* NCDO 2715, *B. bifidum* ATCC 29521 and *L. plantarum* NDRI strain 184. Acetate was found to be the major SCFA produced as the end product of xylo-oligosaccharides fermentation.

Table 1. Studies related to XOS degradation by LAB and Bifidobacteria.

Strain able to ferment XOS	Source and xylooligosaccharides (XOS) type	References
B. adolescentis, *B. longum*, and *B. infantis*	A mixture of xylose,xylobiose, xylotriose, and other saccharides	Okazaki et al.(47)
Bifidobacteria	Commercial XOS (DP 2-4)	Hopkins et al.(48)
L. brevis PEL1, *B. adolescentis* VTT E-991436, *B. angulatum* ATCC 27535	Commercial xylan	Crittenden et al.(2)
L. acidophilus (swine faeces isolate), *B. adolescentis* ATCC 15703, *B. longum* ATCC 15707	Plant cell wall polysaccharides-XOS	Van Laere et al.(22)
Leu. lactis SHO-47, *Lc. lactis* SHO-54, *Lc. lactis* IO-1	Hydrolyzed birch wood xylan	Ohara et al.(26)
B. adolescentis CECT 5781, *B. longum* CECT 4503, *B. infantis* CECT 4551, *B. breve* CECT 4839	XOS obtained from rice husk liquor	Gullon et al.(49)
isolates of *Weissella cibaria* and *W. confusa*	Hydrolyzed birch wood xylan	Patel et al.(16)

Abbreviation: B.- *Bifidobacterium; L.-Lactobacillus; Leu.-Leuconostoc, Lc.- Lactococcus, W. -Weissella*

Recently, we reported utilization of hydrolyzed birch wood xylan from novel probiotic isolates of genus *Weissella* (16, 24). Total six isolates of genus *Weissella,* belonging to either *W. confusa* or *W. cibaria* were checked for growth on hydrolyzed birch wood xylan by optical density measurements in microtiter plates and secondly in batch cultures also confirming concomitant decrease in pH. Out of six, four strains namely 85, 92, 145 and AV1 showed growth in hydrolyzed xylan with remarkable decrease in pH. Analysis of XOS before and after growth established consumption in the DP2 - DP5 range in the four XOS-fermenting strains. XOS were consumed simultaneously with glucose, while xylose was consumed after glucose depletion. The SCFA profile revealed that lactate and acetate were the major SCFA produced as the end product of xylo-oligosaccharides fermentation while propionate or butyrate production was below the detection level and could not be confirmed (16).

Mechanism of XOS Degradation and Utilization : The ability of bifidobacteria to metabolize XOS depends on the efficiency of their xylanolytic enzyme systems. Figure 1 demonstrates the degradation of various XOS and their end products. One xylosidase and a few arabinosidases have been purified and characterized from bifidobacteria. They are *â*-D-xylosidase from *B. breve* K- 110 and arabinosidases from *B. adolescentis* DSM20083 (22) and arabinosidase from *B. breve* (25). Ohara et al. (26) cultivated strains of *Leuconostoc lactis* SHO-47 and *L. lactis* SHO-54 with a hydrolyzed birch wood xylan along with *Lc. lactis* IO-1 and found that xylosidase enzyme of these strains is

localized in their cytoplasm. There are no studies reported on the xylan or XOS fermenting ability of the genus *Weissella* and *Pediococcus*.

In another experimental approach it was found that xylanase activity was higher in the culture broth inoculated with wheat bran oligosaccharides compared to that of Bengal gram husk oligosaccharides; this might be due to the presence of higher DP xylooligosaccharides in the earlier substrate (23). In contrast to that, cell-associated β-xylosidase activity was detected in the XOS fermenting strains of *Weissella* (16). Analysis of genomic data suggests this activity to be linked to genes encoding glycoside hydrolases from family 3, 8 or 43. No endo-β-xylanase activity was detectable.

Biological Properties of XOS as prebiotic and their applications : In general, XOS have been reported to possess antimicrobial activity (27, 28); immunomodulatory activity (30, 31, 32); anti-inflammatory activity (33, 34, 35); and anti-cancer activity (36). The other health beneficiary effects of XOS include antioxidant activity (37), blood- and skin-related effects; anti-allergy, anti-hyperlipidemic effects (17) and applications in cosmetics. Besides biological effects concerning human health, XOS have been employed for phyto-pharmaceutical and feed applications. These properties are mainly attributed to acidic oligosaccharides containing uronic substituents, which can be produced from hardwoods by a combination of enzymatic and/or chemical treatments. There is no any side effect or toxic effect has been reported for XOS during clinical applications still date. The dose of XOS is also not standardized, however like other prebiotic oligosaccharides it is incorporated in milli gram quantities during food or therapeutic application.

a. Anti-tumor activity: Earlier, Hashi and Takeshita (36) reported significant inhibition of the growth of sarcoma-180 and other tumors from glucuronic acid-containing acidic xylans. Conversely, no systematic attempts have been carried out to establish the active principle of

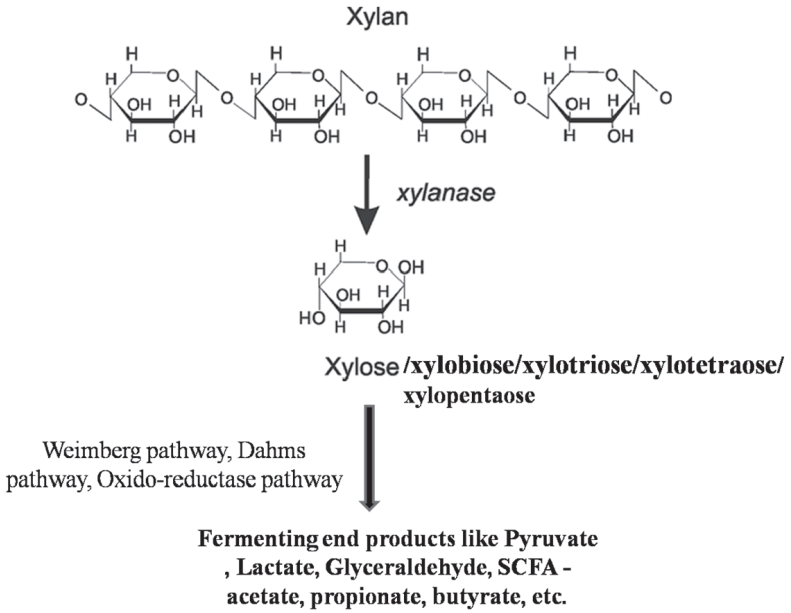

Fig.1. Degradation of Xylan and Xylooligosaccharides in microorganisms with their end products

xylan-type polysaccharides which differ greatly in the type, proportions, and distribution of glycosyl side-chains decorating the â-1,4-D-xylan backbone (4).

b. Antimicrobial activity: Fooks and Gibson (28) reported that *L. plantarum* 0407 and *Lactobacillus pentosus* 905 combined with FOS, inulin, XOS, and mixtures of inulin: FOS and FOS:XOS were effective in inhibiting growth of *E. coli* and *Salmonella enteritidis.* Acidic XOS were produced from birch wood xylan and were tested against Gram-positive and Gram-negative bacteria, *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Proteus mirabilis* and *Helicobacter pylori* (27). In that, aldopentauronic acid proved more active against Gram positive bacteria and *H. pylori.*

Beneficial colonic bacteria such as LAB and bifidobacteria produce carbohydrate degrading enzymes which ferment the NDOs like XOS and produce SCFA such as acetate, propionate and butyrate which provide metabolic energy for the host and help in the acidification of the bowel (11, 38). Acidification can affect the balance of the bacterial species, bacterial metabolic activity and product formation. Probiotic bacteria also shown to exhibit pronounced antibacterial activity against human enteropathogenic bacterial strains (39). Acetate is mainly metabolized in human muscle, kidney, heart and brain, whereas propionate acts as a possible gluconeogenic precursor suppressing the cholesterol synthesis (40). Butyrate is known to have prodifferentiation, anti-proliferation and anti-angiogenic effects on colonocytes (41). Moreover, decrease in pH due to production of SCFA as a result of fermentation correlates with the population growth of the beneficial microbes and in turn inhibits the growth of the undesirable pathogenic bacteria (40, 41).

c. Effect on gastrointestinal related problems: According to Moure et al. (10), administration of 0.12 g of XOS per kg of body weight to male Japanese adults was resulted in beneficial effect *viz.* help to restrain the growth of pathogenic bacteria, to retard disorders caused by imbalanced fermentation in colon, and to avoid intestinal disorders such as constipation, inflammatory bowel disease, diarrhoea, and gastritis. Furthermore, XOS intake has been found to be highly effective for the reduction of severe constipation in pregnant women without adverse effects, and nutritional infant formulas containing XOS have been claimed to have synergistic effects all along the intestinal tract, improving gut barrier maturation.

In comparison with other prebiotic oligosaccharides, the slower fermentation of branched XOS led to higher butyric acid production, which may result in even more advantageous effects, whereas the presence of feruloyl substituents may promote the growth of beneficial bacteria. XOS is used in preparation of micro/nano particles and hydrogels for drug delivery in the treatment and prevention of GT disorders (9).

d. Antioxidant, *antidiabetic and cholesterol reduction activity*: FOS and XOS supplementations significantly increased the activity of antioxidant enzymes – catalase and glutathione reductase – in the blood of diabetic rats (5). FOS and XOS exerted encouraging influences in diabetic rats by significantly improving body weight and reducing hyperglycaemia and cholesterol. Further, the characteristic diabetic complications such as severe glucosuria, proteinuria and advanced glycation end products in renal tissue, diabetic nephropathy, and blood creatinine and urea concentrations were markedly reduced (5). Previously, Imaizumi et al., (42) also reported improved growth retardation, hyperphagia, polydipsia and elevation of serum glucose, TAG and cholesterol in diabetic rats through XOS.

e. Synergistic or prebiotic effects: XOS have been reported to stimulate growth of bifidobacterium in the intestine and thus, they are incorporated in infant foods. Nutritional formula for infants found to own synergistic effects all over the intestine tract and led to improve gut barrier maturation (4). Recently several researchers

have deposited patents on the synergistic effects of probiotic microorganisms together with and prebiotic XOS in synbiotic preparations (43).

f. Immunomodulatory and miscellaneous effects: Recent clinical study established that addition of XOS to feed can increase growth performance, enhance endocrine metabolism, and improve immune function in broiler chickens (44). Furthermore, growth regulatory activities of XOS in aquaculture (45) and poultry (46) were also documented. There were significant differences in the relative weight gain rate and daily weight gain rate as compared with the control in fishes suggesting the usefulness of XOS as a feed additive in the diets of fishes. However, XOS had little influence on the overall bacterial community profile. Other biological effects of XOS alone or as active components of pharmaceutical preparations, cosmetics, exhibit a range of biological activities different from the prebiotic effects related to gut modulation.

Conclusion

XOS have immense prospective as agents to sustain and improve a balanced intestinal microflora for enhanced health and well-being. Available experimental evidences support the hypothesis that XOS and other prebiotics can offer an opportunity to prevent or alleviate gastrointestinal disorders. Even though encouraging results have been obtained for other prebiotics in preliminary clinical trials, the data on XOS are limited. More investigations are required to further elucidate the mechanisms involved in biological effects demonstrated. The properties of XOS offer a new dimension for the development of functional foods. One approach that may be encouraged for future research is the combination of prebiotics and probiotics (as synbiotics). Opportunities exist in exploring the improved knowledge of the synbiotic relationships between colonic microbiota, XOS, and whole body physiopathology.

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Extraction of Genomic DNA from Polysaccharide and Polyphenol Rich Clusterbean (*Cyamopsis tetragonoloba* (L.) Taub.)

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Abstract

Clusterbean (2n=14), also known as guar (*Cyamopsis tetragonoloba* (L.) Taub.) is relatively difficult to work in molecular biology because of the high polysaccharide such as galactomannan content of its endosperm and the high polyphenol content of its leaves. This study aimed to establish a robust genomic-DNA extraction protocol for clusterbean. The efficacy of DNA extraction protocol for a PCR quality genomic DNA extraction was found using DNA fingerprinting. This experiment involves extraction of DNA using a wash buffer containing Tris-HCl, PVP and addition of β- mercaptoethanol separately in each sample and extraction buffer containing CTAB, Tris-HCl, EDTA, NaCl and PVP followed by purification of DNA with RNAase, phenol : chloroform: isoamlyalcohol and finally precipitation of DNA by chilled isopropanol. The protocol is simple and has no special requisites. In terms of quantity (up to1009.5-2119.1 ng/µl) and quality (A260/280 = 1.76 to 1.87) the present method has advantages over many other plant DNA extraction protocols. This protocol can probably be extended also to other leguminous species mainly pulses.

Key words : Clusterbean, Guar, *Cyamopsis,* DNA extraction.

Introduction

Clusterbean (2n=14), also known as guar (*Cyamopsis tetragonoloba* (L.) Taub.), is a major arid legume. The importance of clusterbean lies in its multi utility as vegetable, food, fodder and feed security for both man and animal. However, its economic importance reflects in having a rubber-like substance called galactomannan in its endosperm that has conspicuous wide arrays of industrial utilities (1). Lately, the crop assumed enhanced importance due to uses of galactomannan in fracking process of oil exploration (2). India is the World's largest producer of clusterbean contributing 80 percent to the total global production and the major supplier of guar gum to 65 countries.

The extraction of high-quality DNA is important in any molecular biology work because contaminants such as proteins, polyphenols, and polysaccharides may interfere with enzymes such Taq polymerase in polymerase chain reaction (3,4). Molecular biology studies of clusterbean have been more difficult because present high levels of gum content (galactomannan) in the endosperm and high levels of polyphenols in its other tissues of clusterbean indicate the difficulty in working with this crop (5). Thus, it is important to establish an optimal extraction protocol to yield high-quality DNA and the appropriate part of the plant to use as source. We establish the genemic DNA extraction method from Clusterbean leaves providing high quality genemic DNA and giving good amplification of RAPD primers.

Materials and Methods Plant material

Experimental material comprised twelve genotypes of clusterbean (viz., GG-2; GAUG-0522; HG-75; HG- 365; RGC-471; PRT-15; GG-1; GAUG- 0013; GAUG-9404; FS-277 and PNB) obtained from Centre of Excellence for Research on Pulses, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar. Mature leaf tissue was used in this method. Leaves from one-month-old field grown plants were harvested and brought on ice to laboratory. The leaves were thoroughly washed with tap water and rinsed with distilled water, blot dried and weighed. The leaves were either stored at –80°C or used directly for extraction.

Reagents for DNA extraction

Stocks Solution

To prepare wash buffer and extraction buffer, stocks of reagents were prepared (Table 1) and then subsequent wash buffers and extraction buffers were prepared (Table 2).

Other Reagents

1. β--mercaptoethanol
2. Phenol:chloroform: isoamylalcohol mixture was prepared in the ratio of 25:24:1
3. Chloroform: isoamylalcohol mixture was prepared in the ratio of 24:1
4. 100% chilled isopropanol was used in appropriate quantities.

DNA Extraction

1. The ground fine powder was transferred to 2 ml micro centrifuge tubes and mixed gently by inverting followed by spinning at 12,000 rpm for 3 min at 4^{0}C.
2. The supernatant was discarded and 2 ml of wash buffer was added. The resultant green pellet was mixed with the help of plastic stick.
3. Micro centrifuge tubes were mixed gently by inverting followed by spinning at 12,000 rpm for 3 min at 4^{0}C.
4. The above procedure (wash buffer treatment) was repeated for 2-3 times.

Table 1. Preparation of stocks solution for DNA extraction

Chemical	Preparation
CTAB (10%)	10 g CTAB was added and final volume made to 100 ml with sterile double distilled water.
5 M NaCl	29.22 g of NaCl was added to sterile double distilled water and the final volume was made to 100 ml.
1 M Tris HCl (pH 8.0)	15.76 g of Tris-HCl was added in sterile double distilled water, pH was adjusted to 8.0 by adding pellets of NaOH or 0.1 N HCl and the final volume was made to 100 ml.
0.5 M EDTA(pH 8.0)	It was prepared by adding 18.612 g of EDTA in double distilled water, pH was adjusted to 8.0 by adding pellets of NaOH or 0.1 HCl and the final volume was made to 100 ml.
PVP (10%)	10 g of PVP was added to sterile double distilled water and the final volume was made to 100 ml. After preparation of stocks, all the solutions, except CTAB were autoclaved. From these stocks extraction buffer was prepared by taking following volumes.

Table 2. Components of stock solution for DNA extraction

Stock solution	Components	Quantity
Wash buffer	1M Tris HCl (pH 8.0) 10 % PVP **Sub Total** Double distilled water **Total**	10 ml 10 ml **20 ml** 80 ml **100 ml**
Extraction buffer	10 % CTAB 5 M NaCl 1M Tris HCl (pH 8.0) 0.5M EDTA (pH 8.0) **Sub Total** Double distilled water **Total**	5 ml 20 ml 20 ml 3 ml **48 ml** 52 ml **100 ml**
TE buffer (pH 8.0)	10 mM Tris HCl 1 mM EDTA Double distilled water **Final volume**	0.121 ml 0.03 ml 99.84 ml **100 ml**
RNase a stock (10 mg/ml solution)	10 mM Tris HCl 1 mM EDTA RNase A Double distilled water **Final volume**	**10 µl** **3 µl** 10 mg 987 µl **1 ml**

5. The supernatant was discarded. One ml of extraction buffer was added to the green pallet and mixed with the help of plastic stick.
6. Tubes were maintained at 65^{0}C in a water bath, mixed vigorously and incubated at 65^{0}C for 1 hour with intermittent mixing.
7. After incubation, tubes were centrifuged at 9000 rpm for 7 min at 4^{0}C. The debris was discarded and the supernatant was transferred to a fresh microcentrifuge tubes.
8. Tubes were filled with equal volume of phenol : chloroform : isoamylalcohol (25:24:1) and mixed by gently inverting and later centrifuged at 11000 rpm for 10 min at 4^{0}C.
9. The supernatant was treated with RNase @ 3µl/tube of DNA and incubated at 37^{0}C for 30 min.
10. After incubation, tubes were filled with equal volume of chloroform : isoamylalcohol (24:1) and mixed gently by inverting centrifuged at 11000 rpm for 10 min at 4^{0}C.
11. An aqueous phase was transferred to a fresh microcentrifuge tube and 1 volume of 100% chilled Isopropanol was added to the supernatant and mixed gently for 2 minutes. Thick, bright white pellets of DNA were visible. Tubes were kept at -20^{0}C for over night for better precipitation of DNA.
12. DNA pellet so obtained was centrifuged at 12000 rpm for 10 minutes at 4^{0}C. Pellets were air dried and dissolved in 100µl of Tris-Tris-HCl and (TE) buffer and stored at -20 degree C.

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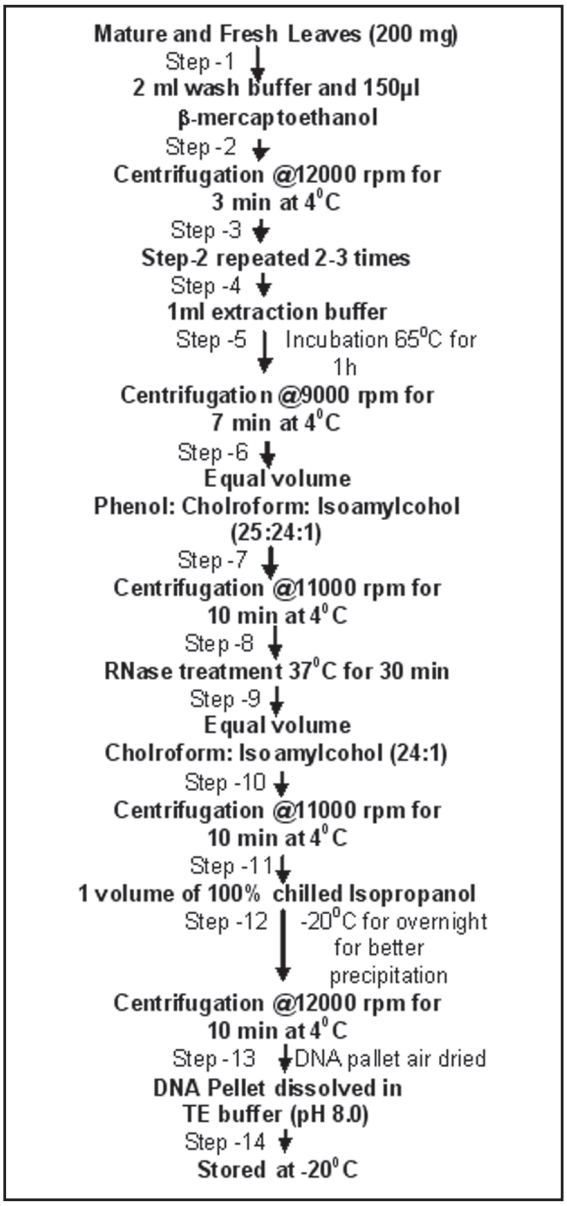
Figure 1. Flow chart in standardized DNA extraction procedure



Quality Check and Quantification of DNA

To estimate the quantity and quality (in terms of protein and RNA contamination) of isolated genomic DNA, spectrophotometery was performed and the data were analyzed using software N.D. (V.3.3.0). For this 2 µl DNA was loaded into the well of Nanodrop spectrophotometer. The concentration of DNA and absorbance were measured at 260 nm and 280 nm. Pure DNA was considered as the one having A260/A280 ratio 1.7 to 1.9 in TE. Strong absorbance at 280 nm resulting in a low A260/A280 ratio indicated the presence of contaminants such as proteins. The nucleic acid concentration was calculated following Sambrook et al. (6).

Agarose Gel Electrophoresis (AGE)

Genomic DNA was resolved by submerged horizontal electrophoresis in 0.8 % (w/v) agarose gel. Gel casting plate was washed with distilled water and dried. Plate was wiped with ethanol and air dried. Agarose gel was prepared by dissolving 0.8 g of agarose in 100 ml 0.5X TBE buffer and heated in a microwave oven. Ethidium bromide (5 mg/ml) 10µl/100 ml TBE buffer was added to it after cooling agarose to approximately 35 Degree C. Gel solution was then poured into the gel casting plate inserted with appropriate comb. After setting of gel, the plate was placed in electrophoresis chamber and submerged using 0.5 X TBE buffer and comb was removed gently. 8 µl genomic DNA samples were mixed with 2 µl of 6 X gel loading dye and were loaded in the wells. Electrophoresis was carried out at constant voltage (70 V for 45 minutes) (9).

Capturing of Gel Image

Agarose gels were photographed by Uvipro Gel Documentation system using UVIgel start MW software version 11.01 and were stored as JPEG files for further analyses.

Optimization of RAPD Reaction

RAPD reaction for clusterbean genotypes was optimized by doing some modification in the method given by Mathews et al. (9). For the optimization of RAPD reaction using DNA extracted from twelve different genotypes of clusterbean, oligonucleotide primers (Operon Technologies Inc. Almeda CA, USA) were used for amplification to standardize the PCR conditions.

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Results and Discussion

Following the procedures described by Doyle and Doyle (9, 10), Murray and Thompson (11) and Dellaporta et al. (12), numerous issues right from cell lysis to DNA separation and a later difficulty in PCR reactions were observed. Amongst those, major problems encountered were low DNA yield and poor amplification reactions in PCR.

Table 3. Quantity and quality of genomic DNA extracted clusterbean mature leaf using modified CTAB extraction method

Genotypes	O.D. A260/A280	Concentration of DNA (ng/µl)
GG-2	1.76	1634.2
GAUG-0522	1.81	1490.1
HG-75	1.77	1156.3
HG-365	1.78	1702.1
RGC-471	1.79	1009.5
PRT-15	1.83	2000.2
GG-1	1.84	1417.3
GAUG- 0013	1.79	1238.2
GAUG-9404	1.87	1395.7
HVG-2-30	1.79	1245.2
FS-277	1.78	1145.9
PNB	1.79	2119.1
Mean	**1.80**	**1462.28**
Average	**1.76-1.87**	**1009.5-2119.1**

The procedure to extract genomic DNA is illustrated in a flowchart in Figure 1. The presented protocol was primarily developed for DNA extraction from maturing leaf tissue of clusterbean, known for its high polysaccharides and polyphenolic content. The extracted DNA was quantified by a spectrophotometric analysis of DNA showed an average concentration of DNA over twelve genotypes as 1462.28 ng/µl with concentration varying in different genotypes from 1009.5-2119.1ng/µl (Table 3). The quality of DNA was analyzed on nanodrop. The DNA evinced absorbance ratio varying from 1.76 to 1.87 at A260/A280 (Table 3). To substantiate the form and purity of extracted DNA sans contaminants, the isolated genomic DNA was gel documented in 0.8 % agarose gel. All the samples of extracted DNA exhibited clear intact band without contaminations (Plate 1).

Plate 1. Genomic DNA of clusterbean genotypes. 1 = 'GG-2', 2 = 'GAUG-0522', 3 = 'HG-75', 4 = 'HG-365', 5 = 'RGC-471', 6 = 'PRT-15', 7 = 'GG-1', 8 = 'GAUG=0013', 9 = 'GAUG - 9404', 10 = 'HVG-2-30', 11 = 'FS-277', 12 = 'PNB'.

The washing method implemented in the extraction process included Tris-HCL, PVP and β-mercaptoetahanol. It is found to be reducing the gumminess in the extracted DNA, which was a major problem in other methods of genomic DNA extraction. This extra method washing step removed the polysaccharides (mainly galactomannon), polyphenols and proteins helping in a successful PCR quality DNA isolation.

Polysaccharide contamination in isolated DNA inhibits enzymatic reactions, such as Taq DNA polymerase amplifications. However, in the present study, successful amplification (Plate 1) indicates the purity and high quality of DNA obtained by our method. This protocol can probably be extended also to other leguminous species mainly pigeonpea, cowpea, kidenybean, lentil, rajama and other pulses. The purity and clean nature of DNA samples could be confirmed in DNA fingerprinting. The utility of the isolated DNA for use in PCR amplification for RAPD profiling was demonstrated with random primers for example OPA 11 and with DNA preparations of all the clusterbean genotypes tested (Plate 2). The method described here is, therefore, rapid, simple and efficient for the isolation of DNA from clusterbean that possess a wide range of activities that can interfere with DNA extraction and analysis.

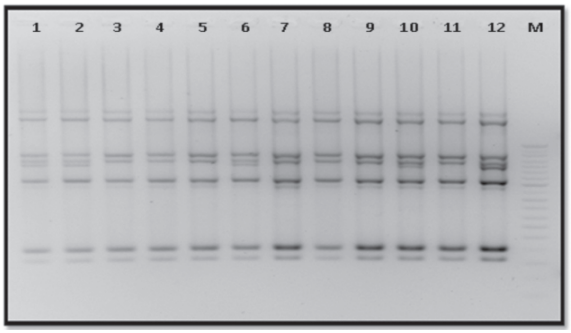

Plate 2. RAPD profile produced by primer OPA 11. Lane M = 50 bp ladder, 1 = 'GG-2', 2 = 'GAUG-0522', 3 = 'HG-75', 4 = 'HG-365', 5 = 'RGC-471', 6 = 'PRT-15', 7 = 'GG-1', 8 = 'GAUG=0013', 9 = 'GAUG- 9404', 10 = 'HVG-2-30', 11 = 'FS-277', 12 = 'PNB'.

Conclusion

An efficient DNA extraction method for clusterbean mature leaf is described. The method consistently produces high yield and high quality genomic DNA at an affordable cost. This method can be used to extract high quality genomic DNA from genotypes of clusterbean mature leaves. The low cost, high throughput, high quality, high yield, and broad applicability of the method make it a useful method for PCR application that need large quantity and high quality DNA.

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The History of Methods of Healing

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Abstract

Different methods of healing that have been developed over time have increased the ability of medicinal professionals to meet the challenges that arise with expansion of their professional roles. The methods of healing of cave people were undeveloped. The methods of Chinese medicine were focused on balancing the internal and external energies. Holistic and sophisticated system of healing represents Ayurveda. Egyptian medicinal texts show a close relationship between the supernatural and empirical healing. Illyrians applied hydrotherapy and physiotherapy. Experiences in the field of religious medicine and initial medicinal institution had Thracians. Healing in Ancient Greece was based on the law of similarity. Greek physicians favored diet and life adaptations in relation to the use of drugs. With the Roman sanitary legislation were regulated public hygiene and sanitation facilities.

Galen made efforts to balance the fluids in the sick person using drugs with opposite nature. Rational drug therapy in Middle Ages decreased and was replaced with Church knowledge. Arabic physicians have rejected the old idea that a bitter pill act best, instead of this they prepared their dosage forms tasty and attractive by silver or gold plating and use of syrups. Paracelsus pioneered in chemically prepared drugs out of raw plants and mineral substances. He used special drug for a particular disease. In the Renaissance, a great jump is made in the preparation of drugs. For a period of about 300 years, significant discoveries were made in the chemistry of drugs. Today, current gene therapy has the potential to prevent, correct, modulate gene or acquired diseases.

Key words: Healing, history, method, drug, idea

Introduction

One of the features of Homo sapiens is a tendency to heal the disease. According to archaeological evidence the need to relief the intensity of a pain is as old as the desire for exploring of new tools. As hardship flint, used for making knives and axes, drugs in nature rarely occur in its most useful form. Active ingredients and medicinal components must first be collected, processed and prepared for inserting in curative form (1).

Introduction to the development of ideas related to drugs, manner of healing and the evolution of the profession increase the ability of medicinal professionals to respond to the challenges that arise with expansion of professional roles.

Healing through the history: In the Stone Age healing is developed spontaneous instinctive, by observing the birds and beasts. The first applications to relieve any pain it was that what was on the immediate man reach like cold water, leaves, earth and mud. Although the healing methods of cave people were undeveloped,

many of today's drugs are used as they were on the availability of prehistoric man (2).

Medicinal plants in the Neolithic period have been recognized and used as food, spices, or spells. Through trial and error, grew folk knowledge about medicinal characteristics of certain natural substances. When healers will be faced with a disease, they have put it in the context of their understanding of the world around them, plenty of good and evil spirits. The healing consisted in the use of beneficial medications with prescribed supernatural significance. Spells of wizards challenged by the magical substances could win diseases. Magical drug beverages were part of the duty of the tribal Shaman healer. Knowledge of this treatment, the shamans kept secret. He (or she) prepared medicines and served as a link between the material and spiritual world. Shaman was usually responsible for all or most supernatural things in the tribe, so they performed the diagnosis and treatment of serious or chronic illnesses. Although primitive people discovered only a small number of effective medications, the concept of the possible impact on body functions through external force, must be considered as one of the greatest human achievements. Holistic approach to healing has taken over many techniques from Shamanism as visualization, altered state of consciousness, hypnotherapy, meditation, positive conception and stress reduction (2,3).

The traditional Chinese medicine offers detailed health care system with a wide range of applications from preventive health care and maintenance, to diagnosis and treatment of acute and chronic disorders. Methods are focused on rational use of internal and external energies employing diet, herbal treatments, acupuncture and breathing techniques (2,3).

Ayurveda (ayus life, ved knowledge) is a complete system of healing which appears in the II millennium in India. Its purpose is to provide a guide towards diet and lifestyle of people in order to remain healthy and the sick people to improve their health. Healing includes herbal treatments (including cinnamon and cardamom), massage, meditation and exercises for posture and breathing, for balancing and forcing the health. Ayurveda observe the interdependence on individual health and the quality of its social life. It is a holistic and sophisticated system where body functioned through the interaction of vital energy, tissues and secreted products (3).

Treatment in ancient Egypt shows greater pharmaceutical improvement with more dosage forms with more complex formulas. Egyptian medicinal texts have a close relationship between the supernatural and empirical treatment. Recommended recipes usually began with prayers or incantations. Herbal remedies were the basic means of treatment with laxatives and enemas as the most important. There were individuals specializing in the collection, preparation and sale of drugs, as was the case with curative practice in Mesopotamia (3).

Illyrians practiced religious, magical medicine and psychotherapy. They believe in more Gods. Medaurus is Illyrian god of drugs and Bindus and Thana were gods of healing mineral springs. Illyrians applied hydrotherapy combined with physiotherapy (3).

Thracians believed in their Thracian's gods. Asclepius was the god of drugs. They had an experience in the field of religious medicine, methods of treatment and in the initial medical facilities (3).

In the earliest records of ancient Greece has been found a similar mixed concept of a drug or pharmakon, word which means magic, drug or poison. Homer (800 BC) in Odyssey underlines the respect for the physicians' wisdom in Egypt, illustrating the outflow and flow of the antic knowledge much earlier than the written word. Early Greek physicians described by Homer, entitled as *demiourgoi,* have progressiveness to diagnose the natural reason for the disease (but they did not rule out the supernatural healing in combination with empirical medicine). Some people, obsessed by persistent pain, traveled to the temple of the god Asclepius. They slept in

the temple in the hope that during the night will be visited by the Asclepius or his daughter Hygeia, which carry a magic snake and jug with curative medication. Rational tradition in the Greek medicine evident by Homer was processed and inserted in the contents of the literature connected with the name of Hippocrates of Cos (425 BC). Based on the benefits acquired from previous natural philosophers i.e. as Thales (590 BC), Anaximander (550 BC), Parmenides (470 BC) and Empedocles (450 BC), Hippocratic physicians formed their rational explanation of the disease. This was achieved through the establishment of a hypothetical relationship among surrounding and humanity, by connecting the four elements air, soil, fire and water with four leading fluids in the body: black bile, blood, yellow bile and phelegm {mucous liquor). Greek - trained physicians (iatros) which has followed by Hippocrates method favored diet and life adaptations in relation to the use of drugs. If these conservative methods are not giving results, the Greek physician prepared his own drugs or would left a prescription to the members of the family to prepare and apply. Healing has been based on the laws of the similarities (2,3).

The health culture of the ancient Macedonian state was folk - empirical. But Macedonian kings showed great interest in its improvement and development. Under the influence of medicine of the previous nations for their protectors are declared the same gods that its supernatural power to treat they handed to the priests, who treated the sick man in temples dedicated to Asclepius. Treatment was performed with psychotherapy, diet fasting, bathing in warm water and massage. Drugs of plant origin were given and were conducted operations for rigging broken bones and dislocated joints. Alexander's Medical School in Alexandria saved ancient Greek medicine from a century of stagnation caused by the appearance of dogmatism. Influenced by the medical thought of the Egyptians and oriental Asian nations, successfully continued the development of natural, rational scientific medicine of Hippocrates, with notable additions of anatomical, physiological, toxicological and surgical knowledge (3).

Romans as a militant people, because of their conquering goals are falling behind in the way of treatment in relation to other nations. Their science and medicine are at lower level. Romans have shown a great interest in the discovery and use of mineral waters for medicinal purposes. Since that time are known the famous Katlanovo spa, Kosovrasti and Banjishte spa near Debar, Kezhovica spa near Stip, Bansko spa near Strumica etc. Roman period is important in health legislation with which were regulated the public hygiene and sanitation objects (4, 5, 6).

Medicine in classical antiquity has reached its top with Galen, so that registrars that followed later insisted on to be compilers and commentators on his work, not highly original thinkers. The impact of Galen was so imbued among medical practitioners that the basis of his medical approach - the balance of the four bodily fluids through various drugs - mixed with folklore and superstition lead the people in their own treatment of the disease. In the western half of the Roman Empire, this medical knowledge has become especially appreciated. Through the Galen's knowledge and writings, who has worked in Rome in the second century of our era, humoral system in medicine obtains the predominance in the following1500 years. Leaving on side the conservative use of drugs of the orthodox Hippocratic physician, Galen created elaborated system through which he made efforts to balance the fluids on the sick man with the use of drugs with expected opposite nature. For example , for medical treatment on outer inflammation, one of the Galen's followers, probably would use cucumber, cold, and moist folk drug, or perhaps the same Galen's follower would tried to cause bleeding, also known as the preferred treatment for the eliminating of the evident blood excess, which cause the disease. Besides the suspected practice of bleeding, Galen represents the use of poly pharmaceutical preparations or to today would be so-called as

"shotgun Recipes ". He thought that the body of the patient will drown out from the complex mixture of substances those which were needed for reestablishing the humoral balance (2, 3).

Middle Ages not made any changes in relation to the manner of medical treatment. As more methods of treatment become alienated of practical experiences more prominent expression of mysticism and superstition was appearing so that medicine in Byzantium represents pale and disfigured image of Greek - Roman medicine. The church became a cultural stabilizing force in the emergence of local feudalism which replaced the centralized government. Rational drug therapy decreased and was replaced with Church's teaching which preached that sin and disease are closely related. Monasteries became centers for healing, both spiritually, and physically, because they essentially are not considered different (2, 3).

At the beginning Arabs totally accepted the authorship of the then known medical manuscripts. However, with the growth of their culture, Islamic medical scholars as Rhazes (860-932 AD) and Avicenna (980-1063 AD) completed the works in the field of medicine. The Arab's invasions brought new drugs and spices in the Centers of Learning. In addition, the Arab's physicians rejected the old idea that the tablets with the bitter taste act best. Instead of this they made a huge effort to prepare their dosage forms more attractive and taste, through coating the pills with silver and gold and the use of syrups. These new more sophisticated drugs demanded more complicated preparation. In this period classic scientific and medical knowledge again prevail in Europe through the Arabic culture (2, 3).

A significant change in the use of drugs to appear, this educational approach ought to be overcome and to approach the suspicious, monitoring methodology. Such drastic changes following the experimental period now called the Renaissance. The time of the Renaissance was mature for the overthrow of the old Galen's concepts of diseases and drugs. New and unknown drugs were arriving from distant lands. Tremendous incentives for these early studies are the discoveries of new drugs in newly discovered countries. As Galen not knew all the diseases of the then known world, and Dioscorides (40-90 AD) and his Arab associates not knew all the drugs. Tobacco, quina, alder buckthorn, ipecacuanha were among the newly discovered drugs from the new world. Galenic physician developed system of balancing the fluids by using drugs with opposite characteristics could not explain the efficiency of quina against malaria. Besides healing malarial fever, quina showed weaker effect in various states followed with fever. After satisfaction the needs of religious books (Bibles, hymns, etc.) appeared a new field of interest among printers, issuing the medical and pharmaceutical works, in which abound and detailed illustrations were of enormous benefit. On the side of medicine, this trend is presented with anatomical best work of Andres Vesalius (1514-1564 AD). Although critical to the progress of medicine, almost-modern, precise works of Vesalius and Fuchs did not affect the treatment of the disease as many speculative, mystical colored literary works of the traveling Swiss surgeon who called him selves as "Paracelsus". Born as Philippus Aureolus Theoprastus Bombastus von Hohenheim in 1493, this medical insurgent well presented the associated behaviors of the common man, the educated physician, practical surgeon and Alchemist. The battles of Paracelsus against the static ideas of Galen, Avicenna and other traditional authorities opened a window into the complicated mind of the Renaissance. He was one of the proponents of chemical prepared drugs from raw plants and mineral substances, but still firmly believes that the collection of these substances should be astrologically determined. At the same time Paracelsus supported the "doctrine of signatures". It is the belief that God set up his sign on healing substances that demonstrates their use in certain diseases. Although Galen could not accept the opinion of the existence of a special medication for certain illness,

Paracelsus propagates it as the only truth. However his efforts for chemically prepared medications stimulate the development of the modern pharmaceutical sciences. Chemical processes, especially distillation, allowed the followers of Paracelsus to isolate healing substances from the drug. Also, the effectiveness of some drugs was clarified. They were involved in professional medicinal practice and were documented in the medical literature. In this period a large jump in the history of pharmacy was the preparation of drugs (2, 3).

The necessary tool in the science - chemistry, was used for preparation of the oldest tools of humankind - medications. Although Paracelsus and his followers criticized the pharmacists, they quickly took up positions on the front of chemistry during the 16th century. For a period of about 300 years, a small number of pharmacists have made significant discoveries in the chemistry of drugs, by isolating many drugs that are still in use and made great contribution to the general knowledge of chemistry. During this time of researching the newly discovered world pharmacists, studied much smaller but equally exciting world in their laboratories. Approaching to this problem in a more contemporary way of seeing these people wanted to isolated pure, crystalline chemical substances which can be chemically identified and quantified. Medicinal plant preparations, no matter how carefully were prepared, show significant differences in strength due to the natural variation of the active ingredients in the plants. The discovery of active principles was not an easy task, and it fascinates pharmacists' researchers for a period of nearly 300 years. To search for the ingredients and to separate, characterize and identify them in the simplest plant was as challenging as any other research. These new, pure active substances with known potency were quickly adopted by the physicians.

This crisis of opinions prompted by the efforts towards further understanding of those advocating for chemical drugs, has supplanted the therapeutic concept of Galenism which lasted for almost 1500 years. Entered a period of about 250 years therapeutic chaos, which lasted until the era of modern pharmacology (2,3).

Today's Ways of Treatment

Conventional (Western) medicine (About 200 years old) : Conventional (Western) medicine was founded on the philosophical opinion of Rene Descartes (1596-1650) who considered the spirit and body separately and on Isaac Newton's (1642-1717) view, presenting the universe as a large mechanical clock which act's in a linear and sequential form. Conventional (Western) medicine considers health as the absence of disease. Also there is an influence of a Darwin's theory (2, 3).

Physicians are trained to treat the defective parts by using drugs, radiation, surgery or to replace body parts. It is better to do something than to build personal power of resistance and ability to overcome the disease.

Conventional (Western) medicine successfully manages acute emergency cases, trauma injuries, bacterial infections and some highly sophisticated surgical interventions. Priority intervention is to resist and overcome the symptoms of the disease, and not the cause. (e.g. The usage of analgetics, anesthetics, anti-inflammatory drugs, antipyretics, etc.). Because the conventional medicine is engrossed with parts and symptoms rather than building the whole operating system, energy, thought and feelings do not well opposes the systemic diseases of long duration (e.g. arthritis, cancer, diabetes, heart disease, hypertension, mental illness and mental etc.).

Today, is actual the gene therapy (drugs and vaccines), a newer class of therapeutics that has the potential to prevent, corrected or modulate the gene or acquired diseases (2, 3).

Homeopathy (from the Greek: homoios-similar and pathos-feeling) : Homeopathy is a therapeutic system about 200 years old developed by German physician and chemist Samuel Hahnemann (1755-1843). It is based on

treating of the disease with small amounts of the substance which in larger amounts will give the same disease symptoms. Available are more than 2000 compounds (2, 3, 7).

Unconventional medicine (so-called Alternative Medicine) *:* Pharmacy primitive skills probably were studied by all those who have dealt with unconventional medicine. It is based on the whole system of mental, emotional and spiritual components that should be considered equally for the human health. Symptoms of the disease are an expression of the wisdom of the body to heal its imbalances and diseases. The concept of treatment is that the own internal healing process exists in each person, time and patience are the main healers of disease (8, 9, 10, 11, 12).

Conclusion

There should not be a conflict in the practicing of Western medicine (allopathic or conventional medicine) and unconventional medicine; on the contrary they should complement each other.

In the case of emergency (appendicitis) Western medicine should be practiced and unconventional medicine is good for prevention and health maintenance.

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Identification and Characterization of Novel Binding Epitope of Tetanus toxoid by Phage Display Peptide Library

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Abstract

Phage display peptide libraries are widely used in various protein-protein interaction studies to determine the immunological binding of proteins and epitope mapping of different targets. In the present study, the peptide library is used to identify the Fab binding epitope site of Tetanus Toxoid (TT). The peptides were screened against Fab by biopanning, using a random peptide 12-mer phage display library. Clones were selected based on the binding activity by phage ELISA and one of the peptide sequence (DTMSYTPNIHLL), which showed highest binding activity towards the Fab was cloned into pGEX-4T$_1$ vector for expression and purified using glutathione agarose affinity chromatography. The binding activity of the purified peptide was demonstrated by ELISA and Immunoblot analysis. Further, analysis of the peptide sequence revealed homology of four amino acids (YTPN) to heavy chain sequence of TT. F13 Fab significantly binds to the YTPN epitope of the TT antigen as indicated by ELISA and may have potential in diagnostics.

Keywords: Tetanus toxoid, Fab, Phage display, Peptide, ELISA.

Introduction

Tetanus is potentially a life threatening disease, caused by a potent neurotoxin viz. tetanus toxin produced by anaerobic bacterium *Clostridium tetani.* Tetanus toxin is a single polypeptide (150 kDa), which consists of a heavy chain harboring a cellular receptor binding domain and a light chain with zinc metalloprotease activity (1-4). Though tetanus is preventable by vaccination and also post-exposure prophylaxis, lack of awareness and timely intervention lead to approximately one million cases of tetanus annually, which results in 0.3-0.5 million deaths globally (5).

Present treatment for tetanus involves usage of animal derived polyclonal antibodies but side effects like serum sickness induced by the host immune response to foreign protein limits its usage. Other limitations of the animal derived immune serum include prolonged immunization procedure, risk of the recipient to certain zoonosis and batch-to-batch variation in the therapeutic efficacy of the antiserum. Advent and usage of recombinant antibodies and peptides to some extent able to overcome the limitations of conventional antibodies. Further, advances in phage display technology where displaying of biologically active materials like peptides or proteins on the surface of bacteriophage particles has facilitated the isolation of specific peptides with affinity towards various targets and potential to serve as prophylactic, therapeutic candidates or diagnostic reagents (6-15).

In the present study, we have utilized the latter approach to identify a peptide, which showed significant binding to F13 Fab using a peptide phage library. The selected peptide was further characterized as a GST tagged protein. The peptide sequence revealed the presence of a four amino acid stretch, also present in sequence of the TT heavy chain and is shown to be responsible for the immune response.

Material and Methods

***Antibodies, bacterial strains, vectors and chemicals*:** The F13 Fab used for epitope mapping was selected using human naïve library. The peptide library kit was purchased from New England Biolabs (NEB) (USA) for selection of peptides. The bacterial strain *E. coli* BL21 (DE3) used for over expression of peptide and vector pGEX-4T$_1$ was purchased from Invitrogen (USA) and GE Healthcare (UK) respectively. The plasmid isolation mini prep kits, DNA-gel extraction kits and PCR purification kits were purchased from Qiagen (Germany). The bacterial strain *E. coli* ER2738 used for the propagation of the recombinant phages was purchased from NEB (USA). Nuclease free water and T4 DNA Ligase were purchased from GeNei (India). Glutathione agarose used for purification of GST tagged proteins and all other fine chemicals used were purchased from Sigma Chemical Company (USA).

***Epitope mapping of anti-Tetanus F13 Fab fragment*:** The F13 Fab antibody epitope was mapped using a linear phage display peptide library of random peptide 12mers fused to a minor coat protein of M13 phage. Briefly, Maxisorp immunotube (Nunc, Denmark) was coated with 100µg/ml of F13 Fab in 0.1M NaHCO3 (pH 8.6) buffer and incubated overnight at 4°C with gentle agitation. The tube was then washed with tris buffered saline containing 0.1% Tween-20 (TBST), followed by addition of blocking buffer (5mg/ml BSA in 0.1M $NaHCO_3$, pH 8.6 containing 0.02% sodium azide) and incubated at 4°C for 1h. The tubes were again washed with PBST and then incubated with $4x10^{10}$ phages in 1ml of sterile tris buffered saline (TBS) at room temperature (RT) for 1h under gentle agitation. The unbound phages were washed out with TBST and the bound phages were eluted with 1ml of 0.2M Glycine-HCl (pH 2.2) and pH neutralized with 150 µl of 1M Tris-HCl buffer (pH 9.1). The eluted phages were infected by inoculating 20 ml of *E. coli* ER2738 culture and then incubated at 37°C with vigorous shaking for 4h. The culture was centrifuged at 10,000 rpm for 10min and the supernatant was transferred to a sterile 50 ml tube. To the supernatant, PEG/NaCl (1/6 volume of supernatant) was added and allowed the phages to precipitate on ice for 1h. The PEG/NaCl precipitate was centrifuged at 10,000 rpm for 15min. The supernatant was discarded and the pellet was dissolved in 1ml of sterile PBS. The suspension was centrifuged at 10,000 rpm for 5min to pellet the residual cells. The supernatant containing phages were re-precipitated by adding 1/6 volume of PEG/NaCl and incubated on ice for 1h followed by centrifugation at 10,000 rpm for 10min. The supernatant was discarded and the pellet was dissolved in 200 µl of sterile PBS. The amplified phages were titrated on LB/IPTG/Xgal plates and used for further rounds of biopanning. The clones obtained after third round of biopanning were checked for binding activity by phage ELISA.

***Phage ELISA*:** Analysis of phage peptides binding to F13 Fab was performed by ELISA using bacterial supernatants according to Phage display peptide library kit instructions. Thirty individual colonies selected after third round of biopanning were inoculated into 3ml cultures of ER2738 cells at mid log phase. The cultures were harvested after incubation at 37°C for 4h and 30 min and centrifuged at 10,000 rpm for 10 min. The pellets obtained were stored at 4°C for phagemid isolation and the supernatants containing phages were collected for analysing in phage ELISA. For phage ELISA, Maxisorp microtiter plates were coated with F13 Fab (300ng/well) and incubated overnight at 4°C. The wells were blocked with blocking buffer for 1h at RT. The plate was washed thrice with TBST and

phages ($1x10^7$) were added and incubated for 2h at RT. The plate was again washed with TBST and incubated with anti-M13 mouse antibody conjugated with HRP (1:6000) for 1h at RT. The bound phages were detected with 3, 3, 5, 5-tetramethylbenzidine peroxidase substrate (Thermo Scientific, USA). The absorbance was read at 450nm using a microplate reader (BIO-TEK, US). The experiment was performed in triplicate and the data shown are the representative of mean and standard deviation.

***Phagemid DNA extraction and sequencing*:** The Phagemid DNA was isolated from the bacterial cells using plasmid purification kit according to the manufacturer's instructions. The DNA was eluted in 50 µl of TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA). The phagemid DNA was sequenced using 96 GIII primer.

***Cloning of F13 Fab binding peptide into pGEX-4T$_1$ vector*:** The peptide, which showed highest binding activity in phage ELISA was selected for cloning and expression in bacterial system. The total DNA stretch of specific peptide (68bp) has been synthesized as two complimentary primers. An equal concentration of complimentary primers of the peptide were taken in 1.5ml tube and denatured at 95°C for 5min. The DNA strands were allowed to renature by auto-annealing. The annealed DNA product and pGEX-4T$_1$ vector were double digested with *EcoR*I and *Not*I restriction endonucleases according to the manufacturer's instructions (NEB, USA) and purified using gel purification kit. The digested DNA fragments were ligated into pGEX-4T$_1$ vector using T4 DNA ligase. The ligated product was transformed into chemically competent TOP 10 cells of *E. coli* strain and plated onto LB agar plates (supplemented with 100µg/ml of ampicillin). The plates were incubated overnight at 37°C and individual colonies were picked from each plate, inoculated into LB medium containing ampicillin (100µg / ml) and cultured overnight at 37°C in an orbital shaker. The overnight grown culture was used for isolation of plasmids by plasmid mini prep kit. The positive clones were confirmed by restriction enzyme digestion with *EcoR*I and *Pst*I. The restriction digestion of pGEX-4T$_1$ vector alone served as a negative control.

***Expression and purification of peptide*:** The positive pGEX-4T$_1$-TTE1 clone was transformed into BL21 (DE3) cells. Individual colonies were picked and grown in LB media containing ampicillin (100µg/ml). The culture was induced with 1mM IPTG once the O.D of the culture reached ~0.6 AU at 600nm. Post induction the culture was grown for 4h at 30°C followed by centrifugation at 5000 x g for 20 min at 4°C to collect the bacterial pellet. The pellet was resuspended in lysis buffer and sonicated. The lysed sample was centrifuged at 10,000 rpm for 30 min and the supernatant was purified by glutathione agarose column chromatography. The bound protein was eluted using reduced glutathione and dialysed against PBS. The dialysed protein was stored at -20°C for further characterization.

***Binding activity of peptide by ELISA*:** ELISA was performed by coating a 96 well microtiter plate (Nunc, Denmark) with 300ng/well of F13 Fab in 50mM carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed thrice with TBST and blocked with 1% bovine gelatin, followed by washing with TBST. The peptide was added to the wells in a two fold serial dilution with the highest concentration of 1 µg /100 µl and incubated at 37°C for 1h. The plate was then washed five times with TBST and binding of the peptide with F13 Fab was detected by addition of anti-GST HRPO conjugate followed by TMB substrate. The reaction was quenched by 1.25M H_2SO_4. Absorbance was measured at 450nm using a microplate reader (BIO-TEK, US). The experiment was repeated thrice to evaluate the concentration dependent binding activity of peptide towards F13 Fab and the data shown are the representative of mean and standard deviation.

***Immunoblot analysis of purified peptide*:** The purified peptide was electrophoresed by 12% SDS-PAGE and immunoblotted onto a PVDF membrane (Hybond-C, GE Health care, USA) using a transblot apparatus (Bio-Rad, USA) following the manufacturer's instructions. The blot was probed with anti-GST peroxidase conjugate (Pierce, USA) and developed using 0.05% 3,3-diaminobenzidine tetra hydrochloride (DAB) (Sigma, USA) and 0.3% hydrogen peroxide in PBS.

***Determination of specificity of peptide by Immunoblot*:** The purified peptide was immunoblotted as mentioned above. The blots were incubated with F13 Fab and *E. coli* lysate respectively and washed thrice with PBST followed by probing with anti-human IgG (Fab specific) HRPO conjugate (Sigma, USA). The blot was developed as mentioned above.

***Indirect ELISA for epitope specificity*:** A 96 well microtiter plate was coated with 200 ng/well of peptide in 50mM carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed thrice with TBST and blocked with 1% bovine gelatin by incubating for 1h at 37°C followed by washing thrice with TBST. The F13 Fab was added in a two fold serial dilution with highest concentration of 2 µg /100 µl and incubated at 37°C for 1h. The unrelated Fab fragments (2µg/100µl of anti-JEV Fab, anti-Rabies Fab and anti-ChikV Fab) were used as a control. The plate was incubated at 37°C for 1h and was washed five times with TBST and dried. The binding activity of the F13 Fab with peptide was detected by addition of His-probe and a chromogenic substrate TMB. The reaction was stopped by the addition of 1.25M H_2SO_4 and the absorbance was measured at 450nm using a microplate reader (BIO-TEK, US). The experiment was performed in triplicate and the data shown are the representative of mean and standard deviation.

Results

***Biopanning against F13 Fab*:** The F13 Fab was screened using human naïve phage library against TT as described previously (16). The Phage display peptide library was used to identify the epitope region of F13 Fab with high binding affinity to TT. Three rounds of biopanning was performed with the library against F13 Fab and the eluted phages exhibited an increase in phage output from round 1 to round 3 of the selections (Table 1) indicating enrichment of F13 Fab specific phage peptides.

***Phage ELISA*:** Phage ELISA shows that there is an incremental enrichment of the specific phages on subsequent rounds of biopanning using the amplified phages (Fig. 1a), whereas no increase in absorbance values was observed for the control (Bovine serum albumin). The eluted peptide phages after three rounds of biopanning were used to infect the *E. coli* ER2738 cells and plated on LB/IPTG/Xgal (Fig. 1b). A total of 30 individual clones were tested for binding by phage ELISA against F13 Fab and amongst them seven clones showed positive binding to F13 Fab (Fig. 1c).

***Analysis of peptide sequence*:** Ten clones from each round of biopanning were selected for sequence analysis. The Phagemid DNA was purified according to the manufacturer's protocol and sequenced. The sequence primer 96 GIII was used for peptide DNA analysis. The DNA sequence was translated and the analyzed peptide sequences are shown in Fig. 2. Amongst them one of the clone, yielded a sequence DTMSYTPNIHLL, which had a possible epitope, viz. TTE1, which was further selected for characterization.

***Cloning of TTE1 into pGEX-4T$_1$ vector*:** The DNA fragment of TTE1 was cloned into pGEX-4T$_1$ vector between *EcoR*I and *Not*I restriction enzyme sites. The plasmid was transformed into BL21 (DE3) cells and the transformed colonies were screened for positive clones by restriction digestion with *EcoR*I and *Pst*I enzymes. A clear mobility shift was observed in the insert as compared to the control on 1% agarose gel. The positive clones yielded a 1047 bp fragment (Fig.

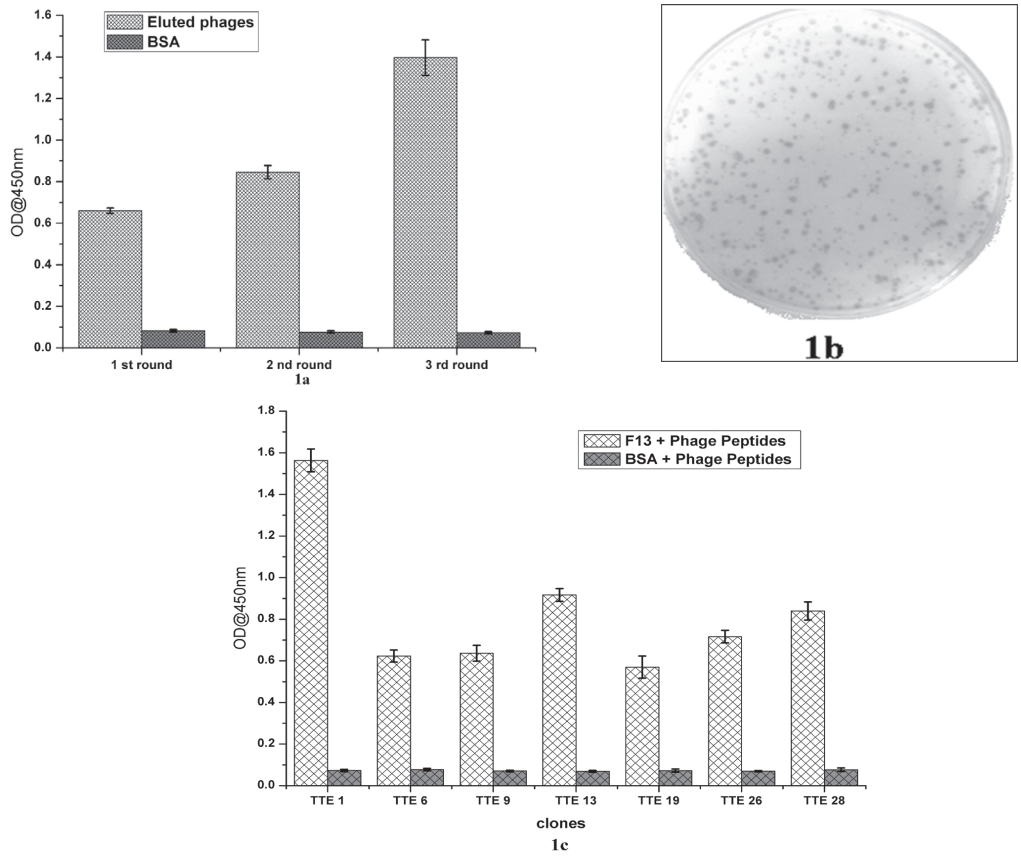

Fig. 1a. Enrichment of F13 Fab specific peptide phages after each round of biopanning. The output phages were incubated with F13 Fab detected by horse radish peroxidase (HRP) conjugated anti-M13 antibody. The BSA served as negative control. The bars indicates the standard deviation of OD values. **1b.** Blue colour plaques are recombinant peptide phages after 3rd round of biopanning infected with *E. coli* ER2738 cells plated on LB/IPTG/Xgal plate. **1c.** Phage ELISA showing the binding activity of different individual peptide phage clones against F13 Fab and BSA used as negative control. The bars indicates the standard deviation of OD values.

1st ROUND	2nd ROUND	3rd ROUND
ALHLYTPPRTPG	DTMSYTPNIHLL	DTMSYTPNIHLL
KLLFAIYLLVPF	MHHRYTPQSSIN	DTMSYTPNIHLL
VAVDNYIRNSSI	SLQARYTPYKNT	DTMSYTPNIHLL
HTWPRSHSTTNR	SNMYTPHHNKPT	WHMSYTPNWSSQ
FYPIKSTHAQPP	AAWEPQAPAPAL	WHMSYTPNWSSQ
WHWNAWNWSSQQ	SNMTQPHHNKPT	FHKNTPTPYYWY
WDRIPTWPYTFS	SHHIAHNLKHRM	HDWRTPNVHHAI
MDINADTSSISQ	VAAPVPPTPRNS	TATAWNGVNDAV
HRLSFRNYSTNY	HLWRQHNVPYAI	LDSNSIFSRGMV
DNAVHTRLLHTG	QIPYTSPTSRAT	TLAVLDTNPHLT

Fig. 2. Sequencing analysis of different clones after each round of biopanning. The enriched peptide sequence after 3rd round is highlighted.

3, Lane 1-7) whereas, the pGEX-4T$_1$ vector alone yielded a 979 bp fragment (Fig. 3, Lane 8) as expected. Positive clones were also confirmed by DNA sequencing and termed as pGEX-4T$_1$-TTE1 (Data not shown).

***Expression and purification of TTE1 peptide*:** The TTE1-GST fusion protein was expressed in *E.coli* and the soluble fraction obtained post cell lysis was purified using a standard glutathione agarose column. SDS-PAGE analysis of the purified protein revealed a band at ~27 kDa (Fig. 4a), which was also detected by anti-GST and F13 Fab in western blot analysis (Fig. 4b, Fig. 4c respectively).

***Indirect ELISA for epitope specificity*:** An indirect ELISA was performed to determine the specificity of F13 Fab towards TTE1-GST

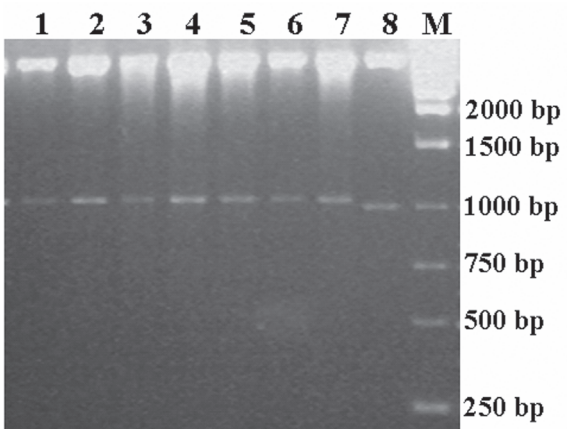

Figure 3. Agarose gel electrophoresis analysis of TTE1 peptide cloned into pGEX-4T$_1$. Lanes 1-7: Positive clones, Lane 8: Vector control, Lane M: DNA standard marker.

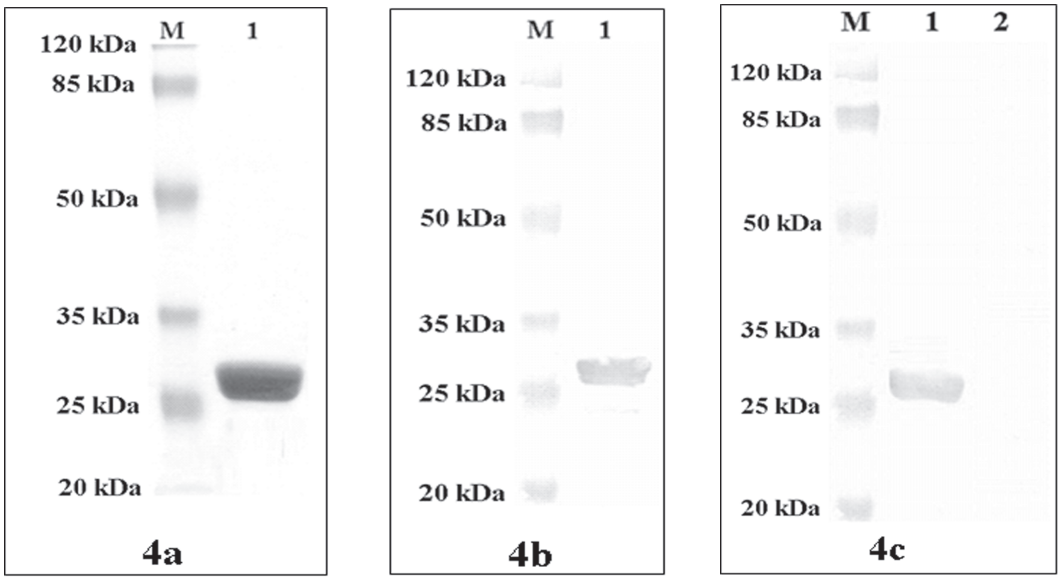

Figure 4a. Detection of purified TTE1-GST protein on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Lane M: Protein molecular size standard. Lane 1 - Purified protein. **4b.** Western blot analysis of purified TTE1-GST protein probed with anti-GST antibody. Lane M: Pre-stained protein molecular size standard. Lane 1: Purified TTE1 protein. **4c.** Reactivity of TTE1 peptide with F13 Fab in immunoblot analysis. Lane M: Pre-stained protein molecular size standard. Lane 1: Purified TTE1-GST protein. Lane 2: *E.coli* lysate as a negative control.

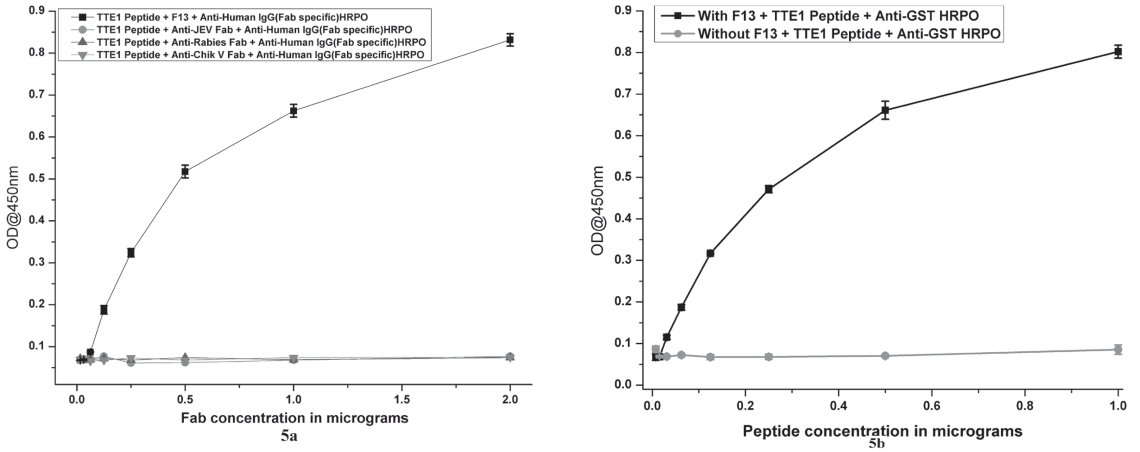

Fig. 5a. Specificity of TTE1 peptide towards unrelated Fabs. The bars indicates the standard deviation of OD values. **5b.** Analysis of binding activity of TTE1 peptide by ELISA to evaluate the activity towards F13 Fab. The bars indicates the standard deviation of OD values.

Table 1. Enrichment of F13 Fab specific clones in three rounds of biopanning.

Number of eluted phages (pfu/ml)			
Library size 4×10^{10}	First round of biopanning 1×10^{3}	Second round of biopanning 2×10^{4}	Third round of biopanning 3.1×10^{5}

peptide. The titration of F13 Fab against TTE1-GST peptide revealed a concentration dependent reduction in the binding signal, whereas unrelated Fabs like anti-JEV Fab, anti-Rabies Fab and anti-ChikV Fab did not show any binding activity to TTE1-GST peptide (Fig. 5a, 5b).

Discussion

There is a vast body of literature where phage display technology has shown to play a vital role in the development of antibody fragments and antibody engineering. A major application of phage display technology is in epitope mapping, which provides information for developing peptide vaccines and diagnostic tools (17-24). Indeed, screening of high affinity peptides from a large diversified phage displayed peptide library against desired target requires less effort, time and resources (25). The choice of biopanning method and strategy is crucial in the selection of binding sequences that are specific for a particular domain.

Tetanus toxin comprises of two polypeptide chains, viz. heavy and light chains connected by disulfide linkage. The heavy chain contains a toxin binding domain and a pH dependent translocation domain, which allows the toxin to bind to gangliosides and enters into cytosol respectively. Whereas, the light chain acts as an endopeptidase which once released in cytosol cleaves synaptobrevin 2, blocking release of GABA and glycine to motor neurons causing prolonged muscle transactions. Tetanus toxin is highly immunogenic and most of the individuals exhibit cellular immune responses to a few identical TT epitopes (26-30). Previous studies identified that tetanus toxin contains multiple epitopes, including some universal epitopes. However, the study based on tetramer guided

epitope mapping revealed the presence of 36 distinct epitopes on the tetanus toxin. Most of the required epitopes, which are presented across the tetanus toxin has been reported to have high affinity towards T cells (31).

The F13 Fab used in this study was successfully selected in our laboratory, using human naïve library against TT. The Fab was expressed as a periplasmic fraction in *E. coli* and purified by affinity chromatography. We used phage display peptide library to identify the epitope of F13 Fab on TT. To maintain the sequence diversity among the binding phage and enrich the specifically bound phages, three rounds of biopanning were carried out. To eliminate unbound phages from immunotube, washing steps were increased progressively 10 to 30 times from first to third round of biopanning. Finally ten clones were selected based to phage ELISA showing highest binding. On peptide sequence analysis, three clones revealed identical sequences whereas, another two had a common YTPN motif. The selected 12-mer peptide sequence was cloned and expressed as a GST-fusion protein in *E. coli*. The ~27 kDa protein was purified to >90% homogeneity using glutathione agarose column chromatography, under non-denaturing conditions. The protein was found to exhibit binding to F13 Fab as indicated by immunoblotting and ELISA, whereas it did not bind to other unrelated Fabs. Indeed, an earlier study by our group F13 Fab was reported to bind to the tetanus toxoid (16).

The analysis of TTE1 peptide revealed the sequence as YTPN from the peptide library and showed sequence similarity with the corresponding amino acid position at 1180-1183 on heavy chain of TT. Further analysis revealed that YTPN was found to be present in one of the predominant CD4 T cell epitope of TT and also shown in sequence YNGLKFIIKRYTPNNEIDSF (TT1170-1189).

Conclusion

The study concludes that a specific epitope in TT has been identified using the F13 Fab by phage display peptide library. The binding activity of the purified peptide was demonstrated by ELISA and Immunoblot analysis. Further, analysis of the peptide sequence revealed homology of four amino acids (YTPN) to heavy chain sequence of TT. F13 Fab significantly binds to the YTPN epitope of the TT antigen as indicated by ELISA. The potential of this peptide as an immunizing agent or diagnostic reagent would be broaden in future.

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Implication of Adrenergic, Serotonergic and Antioxidant systems in the Antidepressant Activity of *Hippophae Salicifolia D.Don*

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Abstract

Depression is one of the most common psychiatric disorders with high mortality, morbidity and economic burden worldwide. Herbal medicines are an important part of the culture and traditions and are widely accepted due to their low incidence of adverse effects. *Hippophae salicifolia D.Don* (HS) is widely used in northern Himalayan region as food, fuel, medicine, veterinary care, cosmetics, agricultural tools and bio-fencing. Berries of this plant have rich micronutrients with potent antioxidant potential. The present study is designed to study potential use of berries in mental depression. HS extract 250 and 500mg/kg and imipramine 25 mg/kg was administered to rats for 7 days. HS extract decreased the immobility time in Tail Suspension Test and Forced Swim Test on par with the standard drug Imipramine, without any significant change in spontaneous motor activity. HS increased the brain dopamine (DA), serotonin (5-HT) and norepinephrine (NE) levels and inhibited the activity of monamine oxidase (MAO-A) concomitantly, it also increased the brain glutathione levels (GSH) and decreased brain lipid peroxides (MDA). The results of the present study indicate that HS shows antidepressant activity in part by increasing the synthesis of NE and by inhibiting the metabolism of NE and 5HT and also by inhibiting lipid peroxidation due to potent antioxidant potential.

Key words: Antidepressant activity, *Hippophae salicifolia* berries, monoamines, antioxidant activity

Introduction

Depression is one of the most common psychiatric disorders with high mortality, morbidity and economic burden worldwide (1, 2). Stressful events are the precipitating factors for the onset of depression (3). Dysfunction in the neurotransmitter levels result in the systemic effect with hyper activation of hypothalamic pituitary adrenal axis (HPA) besides psychological and behavioral consequences which result in hypercortisolemia causing a wide array of organ and immune changes (4). Depression is usually treated with the antidepressant drugs, which cascade serious side effects. So, globally there is greater interest in herbal remedies.

Herbal medicines are an important part of the culture and tradition. Today, most of the population is reliant on herbal medicines for their health care needs. Apart from their cultural significance, this is because herbal medicines are more accessible and affordable (5). Traditional knowledge helps scientists to target plants that may be medicinally useful (6).

In ancient Greece, leaves of sea buckthorn were used as horse fodder for

improving weight and shiny hair, thus gaining the sea buckthorn genus a Graeco-latin name 'Hippophae' (Hippo-Horse, Phaos-to shine). It belongs to family Elaeagnaceae. *Hippophae salicifolia (HS* plant is traditionally utilized by local people of Central Himalaya in multidimensional aspects as food, fuel, medicine, veterinary care, cosmetics, agricultural tools and bio-fencing. HS berries have high content of natural, potent antioxidants including: Ascorbic Acid (Vitamin C), Tocopherols (Vitamin E), Carotenoids, Flavonoids- isorhamnetin, quercetin, ω-3, ω-6 fatty acids, Kaempferol, Catechins, Proanthocyanidins and Chlorogenic Acids (7) and rich in mineral elements such as nitrogen, phosphorus, iron, manganese, boron, calcium, aluminium, silicon and others. Potassium plays an important role in the ionic balance and helps in maintaining the tissue excitability of the human body (8). *Hippophae salicifolia* has attracted a great deal of attention from scientists and engineers all over world due to its concentrated ecological and socio-economical benefits. The present study aims at evaluating whether the edible fruit HS can also serve as an antidepressant.

Material and Methods

Hippophae salicifolia berries extract was commercially procured from the Chansha Organic Herb Inc., China. It was subjected to various chemical tests in order to detect the presence of different phytoconstituents (9).

Animals: Adult healthy Swiss Albino mice of either sex (20-30g) were used. The animals were acclimatized for the laboratory conditions for a period of ten days i.e. room temperature (27±3°C), relative humidity (65±10%), and 12h light/dark cycle. All animals were fed with rodent-pellet diet and water was allowed *ad libitum* under strict hygienic conditions. The experiment was conducted after prior approval from Institutional Animal Ethical Committee (No.1677/PO/a/12/CPCSEA/21).

Acute toxicity study: The acute toxicity study was conducted as per the OECD guidelines 423. Observations were made and recorded systemically 1, 2, 4 and 24 h after dose administration for skin changes, morbidity, aggressiveness, sensitivity of the sound and pain, as well as respiratory movements.

Experimental protocol for antidepressant activity: Mice were randomly divided into 4 groups with six animals in each group. Group I received only vehicle (distilled water, daily) and served as control; Group II received standard antidepressant drug- Imipramine (25mg/kg p.o, daily); Group III received HS lower dose, 250mg/kg/p.o, daily. Group IV received HS higher dose, 500mg/kg/p.o daily. All the animals were received their respective treatment orally by gavage once daily for 7 days. At the end of experimental period (7 days of treatment) the animals were sacrificed by cervical dislocation to obtain brain samples for further studies.

Test for locomotor activity: The locomotor activity was measured using Actophotometer. It consists of cage which has 30 X 30 X 30 cm, and at the bottom six lights and photocells were placed in the outer periphery of the bottom in such a way that a single mouse blocks only one beam. Photocell is activated when the rays of light falls on photocells, the beam of light is interrupted as and when animal crosses the light beam, the number of interruptions were recorded for a period of 5 minutes (10).

Tail suspension test: The total duration of immobility by tail suspension was measured according to the method of Steru et al., (11). Mice both acoustically and visually isolated and suspended 50cm above the floor by adhesive tape placed approximately 1cm from the tip of the tail, immobility time was recorded during a 15 minutes test for animals of all groups.

Forced swim test:FST is the most widely used pharmacological *in-vivo* model for assessment of antidepressant activity. In this model, mice were forced to swim in condition from which they cannot escape and rapidly become immobile,

floating in an upright position and making only small movements to keep their heads above water. The development of immobility reflects the cessation of persistent escape directed behavior or learned helplessness, and a decrease in the duration of immobility, is interpreted as an antidepressant like effect. Mice were placed individually in a glass cylinders (height: 21 cm, diameter: 14.5 cm) containing 15 cm of water at 23 ± 1^{0}C. First 2 min were allowed for acclimatization and the duration of immobility during 4 min was recorded (12).

Estimation of neurotransmitters: Mice were sacrificed after the treatment period (7 days) by decapitation and brains were rapidly removed. The brains were placed in 800µl of ice-cold 0.1M perchloric acid. Individual brain samples were homogenized and centrifuged at 20000 x g at 4^{0}C and stored in a dark freezer at -70^{0}C until further analysis. The pellets were dissolved in 10mM NaOH solution for protein determination using the Bradford protein assay (13). The homogenates were used for the estimation of monoamines like Noradrenaline (NA), Dopamine (DA) and Serotonin (5- HT) according to the method of Alburges et al., (14).

Estimation of Monoamine oxidase A and B: Brain tissue was homogenized in ten volumes of cold sodium phosphate buffer (200mM, p^H-7.4) containing 320 mM sucrose, at 4^{o}C for 30 seconds, using a Teflon glass homogenizer. The homogenate was centrifuged at 600g for 10min at 4^{o}C to remove nuclei and further used for the estimations of MAO-A and MAO-B according to the method of Zheng and Liu (15) and Zhou et al. (16).

Antioxidant studies: Brain tissue samples were homogenized in 50 mM phosphate buffer (pH-7.0) containing 0.1 mM of EDTA to give 5% w/v homogenate. The homogenates were centrifuged at 10000 rpm for 10 min at 0^{o}C in cold centrifuge, filtered and the resulting supernatant was used for further studies.

Lipid peroxidation estimation: MDA level was measured according to the method of Ohkawa et al., (17) at room temperature. 200 µl of supernatant was added to 50 µl of 8.1% sodium dodecylsuphate, vortexed and incubated for ten min at room temperature. 375 µl of thiobarbituric acid (0.6%) was added and placed in a boiling water bath for 60 min and then the sample was allowed to cool to room temperature. A mixture of 1.25ml of butanol:pyridine (1.5:1) was added, vortexed and centrifuged at 1000rpm for 5min. the colored layer (500µl) was measured at 532nm on a Spectrophotometer. The values were expressed in nmoles of MDA formed for mg protein/hr/min.

Reduced glutathione assay: Reduced glutathione was measured according to the method of Ellman, (18) at room temperature. 0.75 ml of supernatant was mixed with 0.75 ml of 4% sulfosalicylic acid then centrifuged at 1200 rpm for 5min at 4 ^{o}C, from this 0.5 ml of supernatant was taken and added to 4.5 ml of 0.01 M DTNB and absorbance was measured at 412 nm using a UV-Visible Spectrophotometer (18).

Statistical analysis: All the data was expressed as mean ± SEM. Differences in mean values between groups were analyzed by one – way analysis of variance (ANOVA) followed by Dunnett's test.

Results and Discussion

The preliminary phytochemical analysis reveals that the hydroalcoholic extract of *HS* showed positive results towards tannins, phenolic compounds, Flavonoids and sugars. The present study is the first, to our knowledge, to show antidepressant-like activity of *HS*, as determined by the forced swimming test (FST), tail suspension test (TST). Tail suspension test and forced swim test are the widely used animal models of depression for the screening of antidepressant activity (11,12). The forced swimming and tail suspension-induced state of immobility in animals claimed to represent a condition similar to human depression and

amenable to reversal by antidepressant drugs. It has been demonstrated that antidepressant drugs reduce this behaviour of abandonment in mice (19). In addition, several extracts from plants have been evaluated in this model with positive results (20-22). Animals are placed in an inescapable situation and the antidepressant-like activity is expressed by the decrease of immobility when compared with control groups.

In the present study we provided convincing evidence that the HS extracts administered by oral route produced specific antidepressant-like effects in TST and FST after 7 days treatment. The results presented here showed that extract at 250 and 500 mg/kg lead to a significant reduction in the immobility period after 7 days treatment in TST in a dose-dependent manner (Table 1, Table 2). In the present study we used mouse as animal and utilized FST and TST for the evaluation of antidepressant activity of HS. In our study, *HS* showed no change in locomotor activity at doses that produced antidepressant-like effect, indicating that the specific actions of this extract on the behavioral model are predictive of antidepressant activity (Table 3).

It is well accepted that increasing brain monoamine neurotransmitters is an effective way to treat depression (23, 24). The dysregulation of the neurotransmitters noradrenaline, serotonin and dopamine has been suggested to play a role in the pathogenesis of depression (25, 26). Generally, the most widely accepted hypothesis of the biological basis of depression implicate serotonin and noradrenaline system dysfunction.

In the present study, to probe the mechanism of action of HS, the effect of HS extract was studied on the brain NE, DA, serotonin levels (Fig 1A, 1B, 1C) and on the activity of MAO-A and B and on the indicators of oxidative stress (GSH, MDA). HS at higher dose

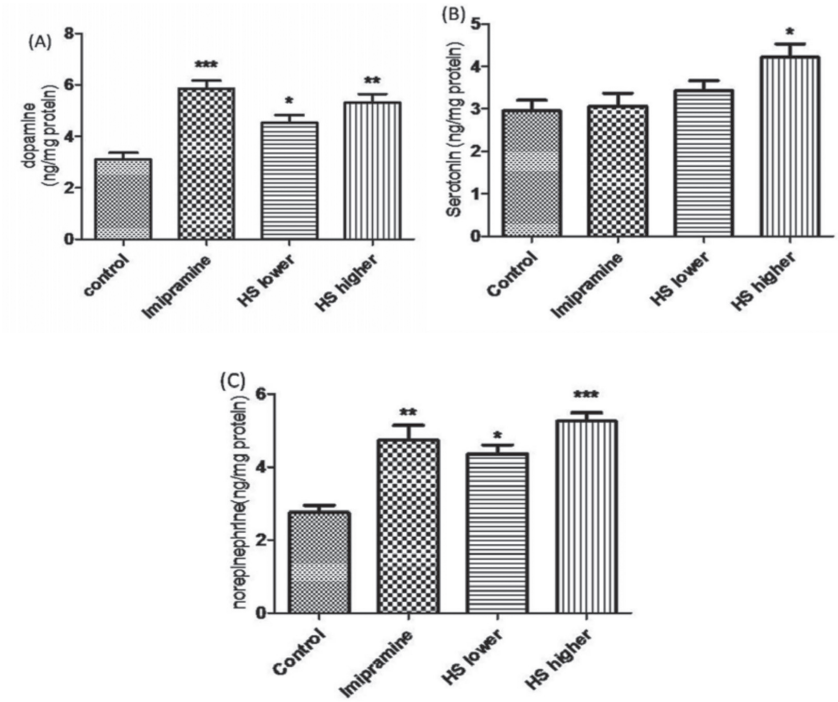

Fig. 1. Effect of HS on (A) dopamine levels (B) Serotonin levels (C) Norepinephrine levels. Values are expressed as Mean ± SEM [n=6]; *(P<0.05), **(P<0.01),***(P<0.001) vs control group

of 500 mg/kg significantly increased the NE, serotonin and DA. HS significantly increased the level of DA and NE with a significant decrease in the activity of MAO-A indicating increased NE is due to increased synthesis as DA levels are increased simultaneously with decreased metabolism as MAO-A activity is decreased. In addition there was no inhibition of MAO-B activity, indicating raise in DA is not due to inhibition of its metabolism (Fig 2A, 2B).

The dopaminergic system is also an important target implicated in the regulation of depression (27). DA involved in brain functions of behaviour, memory etc (28). A number of studies consistently reported a low DA and/or DA metabolite levels in patients with depressive illness (29, 30). In addition, it was demonstrated that chronic treatments with antidepressants such as amineptine improved the dopaminergic neurotransmission, which contribute to therapeutic effect of these drugs (31). HS increased the DA levels on par with standard drug imipramine, indicating the involvement of dopaminergic system in part in the antidepressant activity of these extracts on one hand on the other may be these increased DA levels responsible for the increase in NE levels as DA is precursor for the NE synthesis.

Recently, oxidative stress is closely correlated with depression. Increased lipid peroxidation (32, 33) and decreased antioxidant enzyme levels is reported in depressed patients (34) and preclinical studies have suggested that antioxidants have antidepressant properties (35). The reactive oxygen species (ROS) like hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide, produced during normal cellular metabolic functions, produce oxidative damage in brain (36, 37). The brain is more vulnerable to oxidative stress because of its elevated consumption of oxygen and the consequent generation of large amounts of ROS.

Lipid peroxidation (LPO), an index of

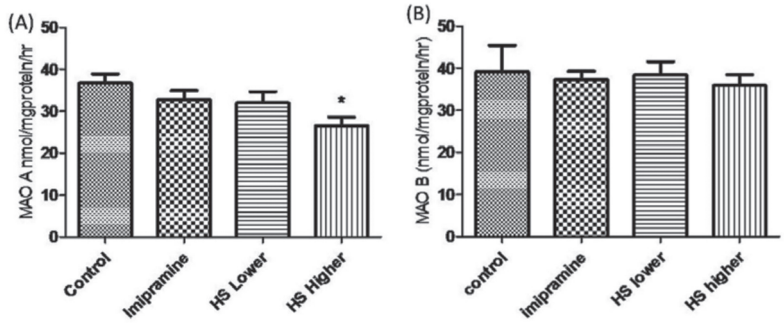

Fig. 2. Effect of HS on (A) MAO-A levels (B) MAO-B levels. Values are expressed as Mean ± SEM [n=6]; *P<0.05 vs control group

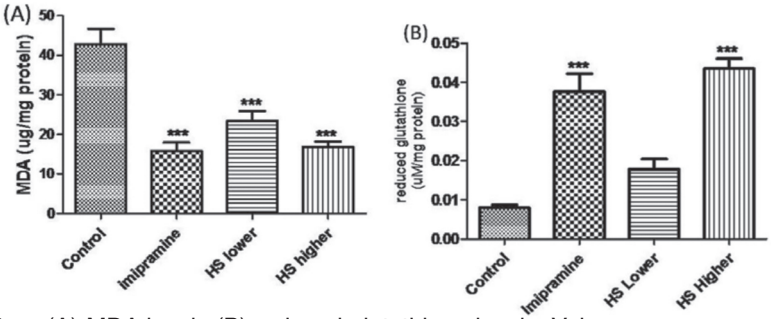

Fig. 3. Effect of HS on (A) MDA levels (B) reduced glutathione levels. Values are expressed as Mean ± SEM [n=6];*** (P<0.001) vs Control group

Table 1. Effect of HS on Immobility time in TST.

	Immobility time in sec			
Groups	**0th Day**	**1st Day**	**3rd Day**	**7th Day**
Control	453.4±9.9	486.2±4.6	512.8±8.1	625.2±6.7
Imipramine	444.7±7.2	373.9±8.7***	223.5±3.8***	87.4±5.7***
HS lower	458.1±6.3	410.5±7.3***	362.2±5.9***	274.5±4.2***
Hs higher	461.3±8.5	336.0±6.7***	250.2±5.4***	123.6±4.9***

Values are expressed as Mean ± SEM [n=6] ***P<0.001 vs control group

Table 2. Effect of HS on Immobility time in FST.

	Immobility time in sec			
Groups	**0th Day**	**1st Day**	**3rd Day**	**7th Day**
Control	59.8±7.3	59.2±5.2	82.6±7.2	101.4±5.8
Imipramine	61.7±5.9	48.4±7.1***	39.5±4.7***	25.6±6.1***
HS lower	63.2±8.5	57.2±6.9***	50.5±6.9***	37.8±5.5***
Hs higher	60.5±7.4	50.9±4.8***	43.1±8.4***	29.8±7.2***

Values are expressed as Mean ± SEM [n=6] ***P<0.001 vs control group

Table 3. Effect of HS on Spontaneous Locomotor activity.

	Score			
Groups	**0th Day**	**1st Day**	**3rd Day**	**7th Day**
Control	129.2±4.2	117.6±9.0	99.2±6.6	78.7±7.3
Imipramine	134.3±3.9	119.9±3.2	126.7±6.9	132.4±3.6
HS lower	121.9±5.6	120.2±3.8	122.3±11.4	124.8±7.9
Hs higher	126.2±5.4	124.3±3.2	123.4±2.4	128.2±4.5

Values are expressed as Mean ± SEM [n=6]

oxidative stress, damages the cell membrane (membrane fluidity, receptors, and ion channels) (38), which may result in calcium influx and cause cell death. In the depressed animal models (FST) as well, decrease in antioxidants and increase in lipid peroxidation was observed**.** In the present study also, depressed rats showed increased lipid peroxidation (Fig. 3A) and decreased reduced glutathione (GSH) (Fig 3B). The increased level of LPO observed in depressed (control) rats, indicates an excessive formation of free radicals and activation of LPO system.

Interestingly, our results evidenced a parallel increase in GSH, the most important antioxidant in response to treatment with extracts in depressed animals. The increase in activity

may provide an effective defense from the damaging effects of not only superoxide anion and hydrogen peroxide but also from damaging and highly reactive hydroxyl radical generated by Fentons reaction (39).

Conclusion

From our results, it can be concluded that HS showed antidepressant activity. The antidepressant like activity of *H. salicifolia* extract might be in part due to increasing the levels of noradrenaline and serotonin by increasing their synthesis and by inhibition of their metabolism by MAO-A and also due to its potent antioxidant potential.

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Molecular Validation of Quality Trait Linked Markers for Exploring the Wheat Germplasm of Eastern Gangetic Plains of India

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Abstract

To fulfill the requirement of wheat consumer's and their changing dietary habit, improvement of wheat quality is necessary. Therefore, breeding for quality parameters along with grain and cooking quality is pre-requisite for any breeding programme. Among the quality parameters grain protein content (GPC) and test grain weight (TGW) have essential role in nutritional value, market value and yield. In this context, GPC and TGW associated gene/ QTLs based markers were validated in a set of 14 and 8 wheat lines/ genotypes of Eastern Gangetic Plains respectively. Out of several markers reported to be associated with *Gpc-B1* gene/ QTLs only Xucw108 and Xucw109 found useful to discriminate GPC and non GPC lines/ genotypes. Similarly, out of four markers tested Xgwm297 was found informative to distinguish between high and low TGW lines. Validation of associated markers in the used 14 and 8 lines, five (Glu1, Glu2, Glu3, Waxwing/Kukuna and PBW343+Glu) were possessing *Gpc-B1* allele and five lines (HUW468, HUW510, Glu3, RS111 and 'PBW343+Glu' lines) had TGW alleles. Interestingly, out of 14 lines, only two lines (Glu3 and PBW343+Glu) had both the alleles and can be used as potential donor lines for transferring both the traits through genomics-assisted breeding.

Key word: *Grain protein content, Test grain weight, validation, Eastern Gangetic Plains (EGP)*

Introduction

Eastern Gangetic plain is a rich resource of germplasm yet to be exploring in the near future breeding programme. Uttar Pradesh, Punjab, Haryana, Rajasthan, Madhya Pradesh, Gujarat, and Bihar are the leading wheat growing state, out of total 80% of the wheat produced by Uttar Pradesh, Punjab, and Haryana. Around 312 wheat varieties were released in six wheat zones since last four decades in India. Notwithstanding, farmers of various zone preferred only few of them, which occupy substantial area and played a major role in increasing productivity (1). Currently, PBW343 is leading and occupied 6 million hectares, while HUW234 largely grown and covered 2–3 million hectares in Eastern Gangetic plains zone (EGPZ) (2). The grain protein content (GPC) is important to decide nutritional value, end products quality (bread, pasta and chapati) and market value of wheat grain, while test grain weight (TGW) is essential with the point of yield (3). Several factors affecting the quality of wheat, like strong inverse correlation of GPC with yield and influence of varying environment (4). As the urbanization patterns changed, end-products of wheat require more quality features like protein (5). Therefore, it is important to combine the high grain yield with

better grain quality to meet the twin challenges of nutritionally superior and high quality wheat products (2). In case of these traits viz. GPC and TGW, early identification of genes/QTLs is prerequisite. Traditionally, these traits are measured by the breeders visually after harvesting, which lengthen time and period of germplasm exploration program due to delay in identification of putative genes/QTLs in the germplasm for their utility.

With the advent of molecular markers, it has been now possible for the breeders to hasten the selection procedure through early identification of responsible genes/QTLs for specific traits at early seedling stage of the plants (3). The DNA based molecular markers are proved to be versatile tools in several aspects like gene tagging/mapping, map based cloning of agronomic important genes, characterization studies, phylogenetic analysis, synteny mapping and marker assisted selection to improve genotypes. These markers not only provide tools to breeders for their different uses but also give them idea to use these identified lines as a different donor in various breeding program (1). To date, several RFLP, CAPS, STS and SSR, linked markers were reported for GPC (6; 7; 8; 9; 13) and TGW (10; 11; 16). There is preponderance of useful genes/QTLs and diverse precious wheat germplasm in India especially in EGPZ and which could be useful in various breeding programme (2). Hence, in our study we emphasized the validation of several molecular markers linked to GPC and TGW in reported germplasm and elite lines to assess the usefulness of published reports of linked markers and identification of suitable genetic material for the target breeding programs.

Materials and Methods

Plant material: The plant materials used for the identification of different useful genes were included popular HUW234, HUW468 and HUW510, Glu2, Glu3 Glu4, Waxwing/Kiritati, Waxwing/Kukuna, K 68, K9107, RS111, 'PBW343+Glu', Kauz Pasture. All the lines were taken in this study on the basis of their prior predictability of traits by the plant breeders. Out of all plant material, HUW234, HUW468 and HUW510 are well adapted high yielding varieties of North East Plain Zone of India in which HUW234 cover 2mha area (2).

DNA extraction: DNA was extracted from 18 days old seedlings by using modified CTAB method (12). The extracted DNA was stored at -20°C for further use.

PCR amplification: The molecular markers used in the present study were selected on the basis of published data. The sequence of primers used to amplify DNA markers for GPC and TGW genes/QTLs are given (Table 1). The PCR amplifications of DNA markers for *Gpc-B1* and TGW were performed in a thermal cycler (Biorad).The polymerase chain reaction was performed in 20 µL volume containing 1 µL of 100 ng µL-1 DNA template, 2 µL of 10 x PCR buffer containing 500 mM KCl and Tris-HCl (pH 8.4, MBI Fermentas, Germany), 0.5 µL of 10 mM dNTP (MBI Fermentas, Germany), 10 pmol of each primer (Metabion, Germany), 0.8 µL of $MgCl_2$ (MBI Fermentas, Germany), 13.5 ìL of double distilled water, and 0.2 µL (5 U µL) *Taq* polymerase enzyme (MBI Fermentas, Germany). For the CAPS marker, PCR and enzyme treatments methodology followed Khan et al. (7).

Results and Discussion

Molecular marker linked to Grain protein content: In this study, we used several reported markers for GPC but only Xucw108 and Xucw109 were found useful. These two markers showed clear difference between GPC and non GPC lines (Fig. 1 and 2). Lines Glu1, Glu2, Glu3, 'PBW343+Glu' and Waxwing/Kukuna were found to have GPC locus while the three prominent cultivars (HUW234, HUW510 and HUW468) of NEPZ were absent for this *Gpc-B1* locus (Table 2). The main drawback of Xucw108 and Xucw109 markers was that, it acted as a dominant marker where it was found to be present in donors while some elite NEPZ varieties showed a null allele. Hence, those lines with the null allele in this study

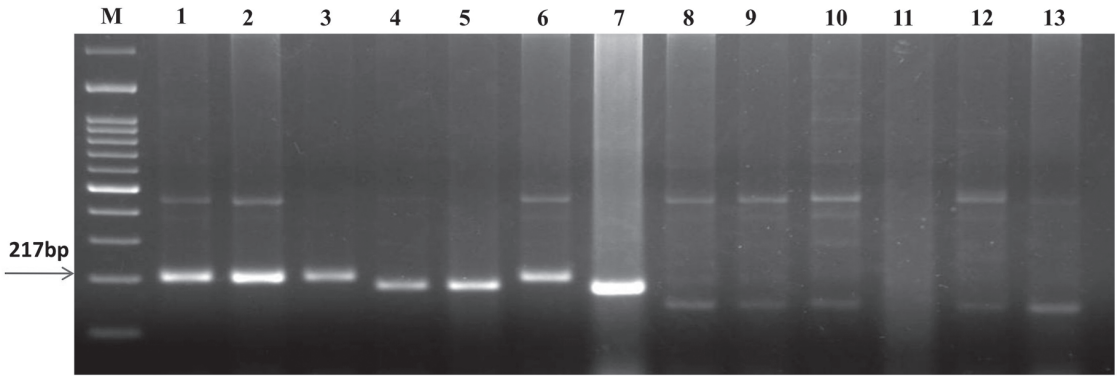

Fig. 1: Molecular profile of Xucw108 marker, M-Ladder 100bp, Parent 1- Glu1, 2-Glu2, 3-Glu3, 4-Glu4, 5-RS111, 6-'PBW343+Glu', 7- Waxwing/Kiritati, 8-Kauz Pasture, 9-K 68, 10-K 9107,11-HUW234, 12-HUW468, 13-HUW510

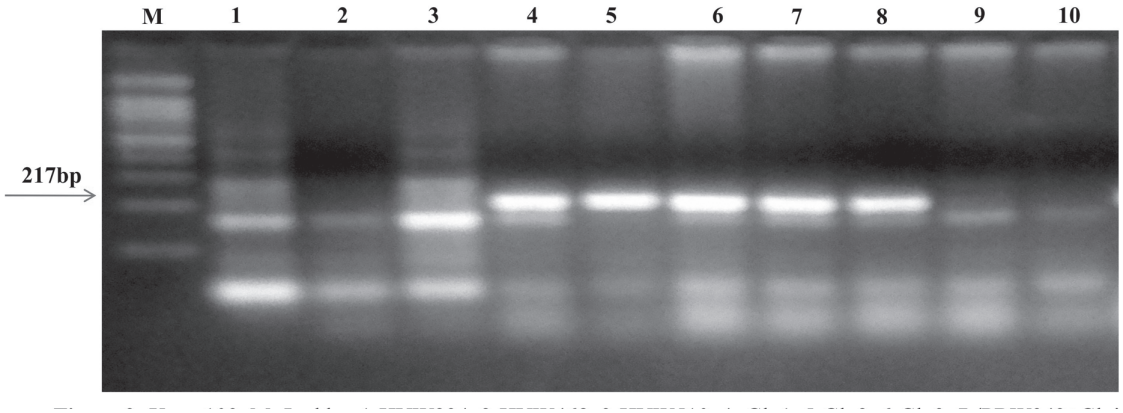

Fig. 2: Molecular profile of Xucw109 marker, M- Ladder, 1-HUW234, 2-HUW468, 3-HUW510, 4- Glu1, 5-Glu2, 6-Glu3, 7-'PBW343+Glu',8- Waxwing/Kukuna, 9-Waxwing/Kiritati, 10-Kauz Pasture

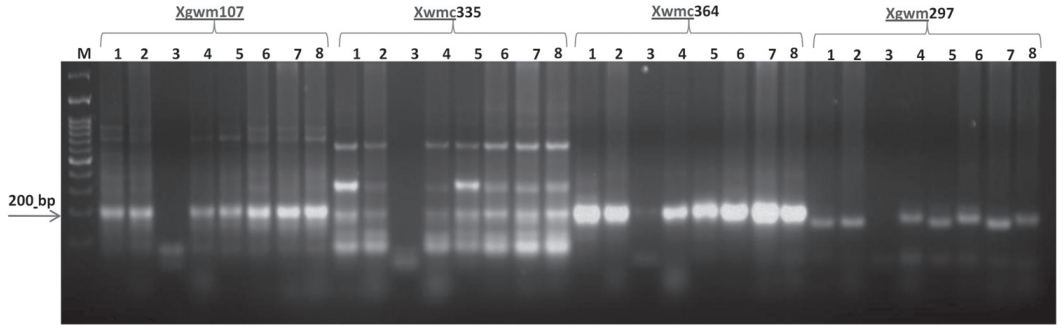

Fig. 3: Molecular profile of TGW linked markers, M -Ladder 100bp,Parent 1-G1, 2-RS111, 3-K 9107, 4-K 68, 5-'PBW343+Glu', 6-HUW234, 7-HUW468, 8-HUW510

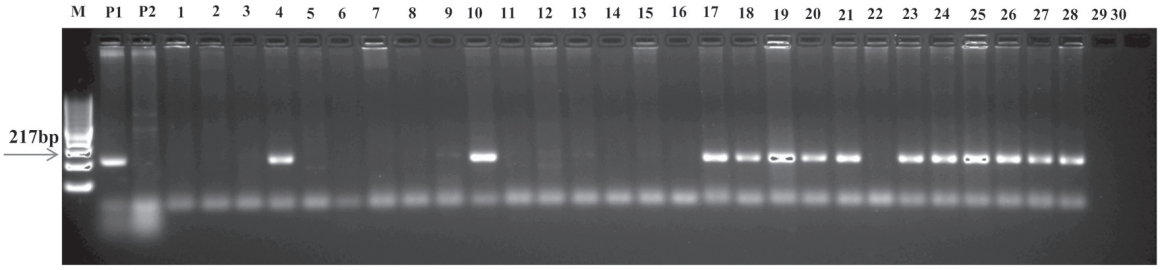

Fig. 4: PCR test Xucw108 (217bp) on HUW510 × Glu1 ILs. M -Ladder 100bp, bands present in *Gpc-B1* positive progenies.

Table 1. List of molecular markers linked to grain protein and thousand grain weight QTLs/genes

S.no.	*Genes/ QTLs*	*Marker/location on chromosome*	*Sequences*	*Investigator*
1	*Gpc-B1*	Xuhw89/6BS	F: 5' TCT CCA AGA GGG GAG AGA CA 3' R: 5' TTC CTC TAC CCA TGA ATC TAG CA3'	Distelfeld et al., 2006
2	*Gpc-B1*	Xgwm1264/2DL	F : 5' TCC CTC TTC CAA GCG CGG ATAG3' R: 5' GGAGGAAGATCTCCCGGAGCAG3'	Prasad et al., 2003
3	*Gpc-B1*	CAPS / XNor-B2 /6BS	F: 5'ATG ATG GTC AAC AAA CGG TGC3' R: 5'TTT ATT GTC ACT ACC TCC CCG3'	Khan et al., 2000
4	*Gpc-B1*	ASA/ XNor-B2 /6BS	F: 5' TTC ACA AAC TAA GGG GAG GGA3' R: 5'CTA CCA TCG AAA GTT GAT AGG GA3'	Khan et al., 2000
5	*Gpc-B1*	Xwmc 415 /5A	F: 5'AATTCGATACCTCTCACTCACG3' R: 5' TCAACTGCTACAACCTAGACCC3'	Singh et al., 2001
6	*Gpc-B1*	Xucw108 /6BS	F: 5' AGCCAGGGATAGAGGAGGAA3' R: 5' AGCTGTGAGCTGGTGTCCTT3'	Uauy et al., 2006
7	*Gpc-B1*	Xucw109 /6BS	F: 5' ATCTGCAATTCCAGGCACAC3' R: 5'CCAGCAGATCAAGGAGAATTG3'	Uauy et al., 2006
8	TGW	Xgwm107 /4B	F'ATTAATACCTGAGGGAGGTGC3' R'GGTCTCAGGAGCAAGAACAC3'	Mir et al. 2012
9	TGW	Xwmc335 /7B	F'TGCGGAGTAGTTCTTCCCCC3' R'ACATCTTGGTGAGATGCCCT3'	Mir et al. 2012
10	TGW	Xwmc364 /7B	F'ATCACAATGCTGGCCCTAAAAC3' R'CAGTGCCAAAATGTCGAAAGTC3'	Mir et al. 2012
11	TGW	Xgwm297 /7B	F'ATC GTC ACG TAT TTT GCA ATG3' R'TGC GTA AGT CTA GCA TTT TCT G3'	Mir et al. 2012

Table 2. The presence or absence of *Gpc-B1* and *TGW* genes/QTLs in EGPZ wheat lines/genotypes

S.n.	Lines/Varieties	*Gpc-B1*	*TGW*
1	HUW234	-	-
2	HUW468	-	+
3	HUW510	-	+
4	Glu1	+	+
5	Glu2	+	*
6	Glu3	+	*
7	Glu4	-	*
8	Waxwing/Kiritati	-	*
9	Waxwing/Kukuna	+	*
10	K 68	-	-
11	K 9107	-	-
12	RS111	-	+
13	'PBW343+Glu'	+	+
14	Kauz Pasture	-	*

+ Present, - Absent, * Not used in study

were lacking *Gpc-B*1. Therefore, these markers are suitable for identifying *Gpc-B1* rich lines. For the confirmation of utility and deployment of these markers, we tested these markers in the 115 introgression lines (ILs) of the cross HUW510 × Glu1, linked marker Xucw108 was used, which amplify 217 bp size band on agarose gel and identified 72 positive progenies (Fig. 4).

Genetic analysis in previous, studies revealed chromosome arm 6BS was identified to linked with GPC quantitative trait locus (QTL) in a population of recombinant inbred lines derived from the *T. turgidum ssp*. durum cultivar Langdon (LDN) and the chromosome substitution line LDN (DIC6B) (8). This GPC locus increased ~14 g kg^{-1} in both tetraploid and hexaploid lines. Meanwhile, various SSR, CAPS and ASA markers Xgwm1264, XNor-B2 and Xwmc 415 were mapped on 2DL, 6Bs and 5A for QTLs QGpc.ccsu-2D, XNor-B2 and QGpc.ccsu-5A1respectively, (7;8). Olmos et al. (13) identified QTL as a simple Mendelian locus, *Gpc-B1*, which was later, located within a 0.3cM interval. Flanking markers Xuhw89 and Xucw71 for *Gpc-B1* flank a 245 kb physical contig (14). Later on this 245 kb DIC segment showed delay senescence and increased GPC in tetraploid and hexaploid wheat lines. Sequencing of this complete region (DQ871219) revealed five genes linked to the *Gpc-B1* locus. Three recombinant substitution lines with recombination events between markers Xucw106 and Xucw109 delimited a 7.4 kb region, Xucw108 falls in between this region (9).

Promising source of genetic variation in protein was wild emmer wheat [*T.turgidum ssp.*

dicoccoides (DIC)] the ancestor of cultivated pasta wheat (*T. turgidum ssp.* durum) (15). In our study, the Glu lines were used for validation the gene *Gpc-B1* was non adaptive genotype for this region, with poor grain texture and chapati quality. Because the *Gpc-B1* allele for high GPC was first identified in wild emmer wheat *T. turgidum* ssp. *Dicoccoides* accession FA15-3 (15). The high GPC gene from *dicoccoides* was transferred to hexaploid wheat. Hexaploid cultivar Glu-pro was developed by Dr. R. Frohberg from a three-way cross between two bread wheat cultivars and the same *dicoccoides* accession used to develop the substitution line LDN(DIC6B) (7).

Molecular marker linked to test grain weight (TGW): Out of four linked markers taken on the basis of its more phenotypic variance, only Xgwm297 was found useful for the study because it showed polymorphism and differentiated the lines with TGW and non TGW (Figure 3). As the results revealed HUW234, K68 and K9107 promising varieties are lacking TGW, while HUW468, HUW510, Glu3, RS111 and 'PBW343+Glu' can be exploited for introgression of TGW traits (Table 2). Though, the TGW in bread wheat having polygenic control, genetically only a few major and many minor QTL found spread over all 21 chromosomes of common wheat (10). That is why through classical methods of plant breeding it seems tough to improve TGW in bread wheat. In recent studies several molecular marker linked to TGW QTLs were identified , out of which three QTLs (QGw.ccsu-1A.2, QGw.ccsu-2B.1, QGw.ccsu-7A.1) on 1A, 2B, 7A and one QTL (QGw.ccsu-7D.1) to a QTL (QTgw.ipk-7D) were similar to previously identified QTLs, later same (QTgw.ipk-7D) region fine-mapped (10;11,16). In other studies (11) revealed five QTLs located on 2A 6A, 1D and 5D. While, Ramya et al. (16) identified 10 QTLs for 1000-kernel weight, were on wheat chromosomes 1A, 1D, 2B, 2D, 4B, 5B, and 6B. In conclusion validation of these reported markers in sets of different lines/varieties of wheat may useful for MAS for pyramiding these traits to improve bread wheat cultivar.

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Production and Characterization of Lipase from Novel Bacterium *Stenotrophomonas maltophilia* RSP-09

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Abstract

Lipase producing strains play key role in the enzymological remediation of oil polluted soils. Diversity of cultivable lipase producing bacteria in the industrial localities of Hyderabad was studied with samples collected from different areas. Among isolated bacterial strains, the highest lipase producing strain was identified both biochemically and 16S rRNA ribotyping. The 16S rRNA studies revealed that the strain belongs to *Xanthomonadaceae* family and identified as *Stenotrophomonas maltophilia*. An overall 3-fold enhanced lipase production (175µmoles/ml) was achieved after improved conditions of production medium. Among used nutrient sources sesame oil and tryptone were found to be the most suitable substrates for maximum enzyme production. The maximum enzyme production was observed at pH 8.0 and at rotation speed of 150 rpm. Na^+ ions induced the enzyme production where as Hg^{2+} ions inhibited the enzyme production. 80 % Ammonium Sulphate precipitation showed maximum lipase activity and the molecular weight of produced lipase was identified 45 kDa based on SDS-PAGE. Optimum enzyme activity was noticed at pH (6.0) and at 50 ºC. The observed Km for this enzyme is 0.727 and showed V_{max} of 156.6µmoles/ml indicating lower Km than reported lipases that confirmed higher affinity of the enzyme for its substrate tributyrin. Arrhenius plot analysis for energy of activation denoted 11.07 $Kjmol^{-1}$ K^{-1} for this lipase.

Key words: Lipases, 16S rRNA, *Stenotrophomonas maltophilia*, Optimization, Ammonium sulphate precipitation, Enzyme kinetics, Activation energy.

Introduction

Lipases (triacylglycerol lipases EC 3.1.1.3) classically employed for hydrolysis of triglycerides with concomitant production of free fatty acids and glycerol (3). Lipases evolved as leading biocatalysts and provide enormous contribution to underexploited lipid technology in industrial applications especially in hydrolysis of triglycerides and inter- and trans-esterification reactions. These biocatalysts also display catalytic activity towards a large variety of alcohols and acids in ester synthesis reactions under hydrophobic environment. Esterification by lipases appears to be an attractive alternative to bulk chemical routes due to their ecofriendly process. Esters of short and medium chain carboxylic acids and alcohols play a significant role in the food industry as flavoring and aromatic agents (4), whereas methyl and ethyl esters of long chain carboxylic acids function as fuel for the diesel engines, and esters of long chain carboxylic acid and alcohols (waxes) have applications in cosmetic formulations as lubricative and additive agents (5).

Lipase producing organisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, etc. Though, they have been isolated, purified and characterized for their catalytic properties from fungi, yeast, bacteria,

plant and animal sources (1), bacterial origin lipases gained importance in various industrial sectors since bacterial sources are more stable and economical in bulk production over animal and plant based (2). Broadly, bacterial lipolytic enzymes are classified as true lipases (3.1.1.3: hydrolyzes long chain fats), carboxyl esterases (3.1.1.1: converts small chain fatty acids to esters) and phospholipases (3.1.1.4: hydrolyzes phospholipids of different nature) (6). Among these, true lipases play significant role at commercial level in hydrolysis of long chain fatty oils, trans- and inter- esterification depending on the catalytic environment pH. This can be exemplified that under neutral pH environment, true lipase acts as hydrolytic enzyme while under acidic environment they catalyse inter- or trans-esterification. Because of multi-catalytic properties of true lipases industrial application web is increasing day by day. In addition, these catalysts catalytic properties differ with source to source especially in Km, Vmax, Kcat, Ea (energy of activation), substrate specificity, etc. These unique properties drove the researcher for isolation and screening the lipase producing microorganisms from virgin nitch for potential lipase producing strains with unconventional properties like alkalophilic, halophilic and thermophilic (7). In view of the above, the present study aimed to isolate and screen lipolytic compelling bacterial strains from oil contaminated soil samples as this is known for bioaugumented nitch area for lipase producing strains. In this we report isolation of novel true lipase producing bacterial strain, *Stenotrophomonas maltophilia* strain belongs to *Xanthomonadaceae* family. The lipase characterization data suggest that the isolated strain produces true lipolytic enzyme which has low Km value compared with literature reports indicating its application potential at various industrial sectors.

Materials and Methods

***Collection of soil samples*:** Soil samples were collected from industrial localities, contaminated with oils, located at outskirts of Hyderabad city. The collected soil samples were stored in sterile plastic bags and brought to laboratory. The samples were sealed in sterile containers and stored at 4 ^{o}C until further use.

***Screening of lipase producing bacteria*:** One gram of soil was taken from each sample which was suspended in 100 ml of sterile distilled water and kept for agitation for 30 min in orbital shaking incubator at 30 ^{o}C and 150 rpm. Serial dilutions were made from these soil suspensions with dilutions ranging from 10^{-1} to 10^{-6}. From each dilution, 1 ml of sample was used as inoculum on tributyrin agar base medium (composition g/L: Peptone 5.0, Yeast Extract 3.0, Tributyrin 10.0, NaCl 1.0 and Agar 20.0) for the developing lipase producing bacteria by pour plate method. The inoculated agar plates were incubated at 37^{o}C. After 48 h of incubation, the plates were observed for clearance zones around the colonies. Colonies which produced clearance zone were freshly streaked on tributyrin agar slants and stored at 4 ^{o}C until use. All the isolates capable of producing lipase were screened further to select the best isolate which produces maximum enzyme based on single streak method. The isolate which produced maximum clearance zone was selected for further studies.

***Characterization of isolated strain*:** The selected isolate (RSP-09) was characterized morphologically, physiologically, biochemically and genetically (16S rRNA Sequencing). Morphological characterization including gram staining, spore staining, and motility tests were performed according to Bergey's manual of determinative bacteriology. Physiological studies such as growth at different pH, NaCl concentration (%, w/v) and different temperatures were performed. The selected strain was analyzed for oxidase, catalase, citrate utilization, nitrate reduction, starch hydrolysis, metabolism of different sugars (Glucose, sucrose, maltose, ribose, raffinose, rhamnose and xylose etc.) and production of H_2S, urease and indole. The molecular identification (16S rRNA sequencing) was performed at Xcelris labs limited, Ahmedabad, India. DNA was extracted from the culture and its quality was evaluated by agarose

(1.2%) gel electrophoresis. A fragment of the 16S rRNA gene was amplified by PCR and purified. Forward and reverse DNA sequencing reactions were carried out with primers 8F and 1492R (Universal primers) using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The sequence obtained was aligned using data software and a phylogenetic tree was constructed based on maximum likelihood method using MEGA 5.0 software.

***Production of lipase enzyme*:** Initially the isolate (RSP-09) was cultured in 50 ml of seed medium (composition g/L: Peptone 10.0, Beef Extract 3.0 and NaCl 5.0 of pH 7.0 at 37 ^{o}C for 24 h at 150 rpm) in Erlenmeyer flask. For bulk production, 0.2% of inoculum from seed medium was transferred to 100 ml of production medium (composition g/L: Starch 20.0, Peptone 20.0, NH_4Cl 3.8, $MgSO_4$ 1.0, K_2HPO_4 5.0 and Tributyrin 2% of pH 7.0 at 37 ^{o}C for 48 h at 150 rpm) in Erlenmeyer flask. Cell mass from the fermented broth was removed by centrifugation process at 10,000 rpm for 10 min at 4 ^{o}C. The supernatant obtained after centrifugation was used as source of lipase enzyme.

***Lipase assay*:** Lipase activity was estimated by titrimetric method using tributyrin as a substrate. Tributyrin (10% v/v) was emulsified with gum Arabic (5% w/v) in 50 mM potassium phosphate buffer (pH 7.0). Crude enzyme sample (100 µl) was added to 1 ml of emulsion and incubated for 15 min at 37 ºC. The reaction was stopped by adding 1 ml of ethanol to the reaction mixture. The fatty acids liberated from the reaction mixture were estimated by titrating against 0.05N NaOH solution using thymolphthalein indicator. The quantity of enzyme produced was expressed in terms of µmoles/ml of fatty acids released from triglycerides.

***Optimization of lipase enzyme production*:** The isolate RSP-09 was initially cultured in seed medium. From the seed medium 0.2% of inoculum was transferred to production medium and incubated at 37 ºC with agitation speed of 150 rpm. Samples were collected from the fermented broth for every 24h and were centrifuged at 10,000 rpm for 10 min at 4 ºC. The supernatant collected at predetermined times was used as crude enzyme solution and was assayed for enzyme activity. To study the effect of pH on the production of lipase enzyme the pH of the production medium was varied from 4 to 10 with an interval of 1 pH without altering other constituents of the medium. Effect of different carbon sources on the production of lipase enzyme was studied by replacing the tributyrin in the production medium with different oil emulsions (coconut oil, ghee, olive oil, triacetin, Tributyrin, Sesame oil, Soya oil, and starch). To study the effect of different nitrogen sources on the lipase production the nitrogen source in the production medium was replaced with different nitrogen sources like beef extract, tryptone, peptone, potassium nitrate, yeast extract, soybean casein digest, meat extract, ammonium sulfate. Effect of inducers/inhibitors on the production of lipase enzyme was studied by addition of different inducers/inhibitors of 0.1% concentration to the production medium. Different compounds such as barium chloride, calcium chloride, EDTA, ferrous sulfate, ferric chloride, mercuric chloride, sodium chloride, PMSF, calcium carbonate were added to the production medium (in predetermined concentrations) to study their influence on lipase production. All flasks are inoculated with 0.2% of inoculum and incubated at 37 ºC with 150 rpm for 48 h. Samples were collected from the flasks at different time intervals and the supernatant was assayed for enzyme activity.

The effect of aeration on the production of lipase enzyme was studied using different volumes of production medium (10 to 50 ml with an interval of 10 ml in 100 ml) in Erlenmeyer flasks and comparing enzyme production at stationary condition and at agitation of 150rpm by inoculating with 0.2% of inoculum and incubating at 37 ºC for 48 h.

***Partial purification and characterization of lipase enzyme*:** Ammonium sulphate precipitation method was used for partial

purification of extracellular lipase enzyme produced by isolated strain, *Stenotrophomonas maltophila* RSP-09. The culture supernatant was subjected to protein fractionation at differential ammonium sulphate saturations. Precipitation of protein was done by addition of small increments of solid ammonium sulphate at 4 ^{o}C with constant stirring to obtain four fractions i.e., 30 %, 50 %, 80 % and 100 %. At the end of each fractionation, the mixture was allowed to stand for 60 min and was centrifuged at 15,000 x g for 15 min at 4 ^{o}C. Pellet was collected and the supernatant was used for further precipitation. The collected precipitate was re-suspended in 0.05 M Phosphate buffer of pH 7.2 and dialyzed against the same buffer for overnight using cellulose acetate membranes (12 kDa cutoff) to remove excess ammonium sulphate. The obtained pellet was checked for enzyme activity and used for determination of molecular weight by Gel electrophoresis.

***Native and SDS PAGE*:** The molecular mass of partially purified lipase enzyme was determined by Native and SDS-PAGE according to the protocol of Laemmli (1970) (9). For non denaturing PAGE and SDS PAGE, 15 % separating gel and 4 % stacking gel were used. Electrophoresis was performed at 100V for 2 hrs. After electrophoresis, gel was removed from the plates and stained with 0.025 % coomassie brilliant blue dye for six hrs and then transferred to de-staining solution (40 % methanol + 10 % acetic acid). The molecular mass of lipase was determined by comparing with the standard protein markers obtained from Genei and SRL Ltd.

***Kinetic studies and Energy of activation (Ea)*:** The effect of temperature on the enzyme activity was performed by incubating the enzyme at temperature range from 30 to 75 ^{o}C with increments of 5 ^{o}C. Similarly effect of pH on the lipase activity was determined by using different buffers of pH ranging from (2 to 13). Effect of substrate concentration (Tributyrin conc. 1.25 to 20%) on the lipase activity was performed at optimum conditions. The data obtained was used for calculation of Vmax (maximum velocity) and Km (Michaelis constant) from Line weaver-Burk and Hofstee and Hanes-Woof plots. Energy of activation was calculated by using Arrhenius plots.

Results

Screening and isolation of lipase producing strains: One of the best nitch for isolation of lipases producing microbes could be the oily environment as most of the lipase producing strains utilize the fatty substrates as carbon and energy source for their metabolic activities. Hence, tributyrin agar was selected as source for identifying the microbial populations that produce lipases in this study. Though several bacterial colonies were appeared on the tributyrin agar plates upon incubation of collected soil at 37^{o}C, only 9 colonies were initially screened based on the zone of clearance on tributyrin agar plates. The selected colonies were purified to single colony by quaternary streaking method for purification and were maintained on nutrient agar slants at 4 ^{o}C until further use. Further, all nine bacterial colonies were screened by using single streak method on tributyrin agar plates and the strain which had maximum clearance zone (Fig. 1) was selected for characterization and production of lipase and designated as RSP-09.

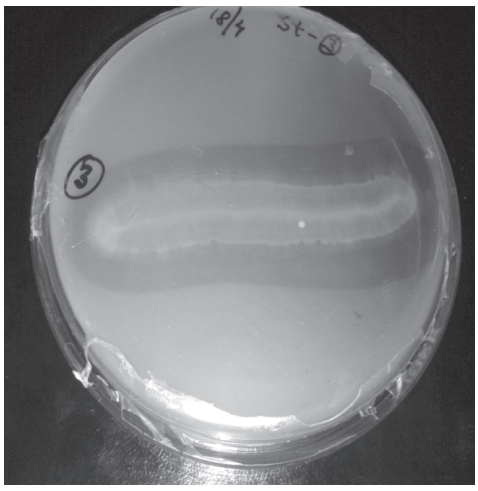

Fig 1. Zone of clearance by RSP-09 on tributyrin agar plate

***Characterization of isolated strain*:** The selected isolate, strain RSP-09, characterized for its morphological, biochemical properties and also for 16S rRNA gene sequencing. Cells of isolated strain RSP-09 were gram negative, rod shaped and motile in nature. Biochemical analysis indicated positive response to catalase, nitrate reductase, urease, gelatin liquefaction, colloidal chitin and casein hydrolysis while citrate utilization test, MR-VP, oxidase, indole production, H_2S production and starch hydrolysis tests were negative for this strain (Table 1). Fermentation of different sugars such as glucose, mannose, galactose, sucrose, cellobiose, raffinose, rhamnose, xylose, salicin, inositol and mannitol was negative (Table 1). The biochemical characterization (Table 1) revealed that the isolate belong to *Xanthomonaceae* family. The 16S rRNA gene sequencing analysis of the isolate yielded 1405 base pairs and NCBI BLAST search analysis showed that the sequence was similar to the sequence of *Stenotrophomonas maltophilia.* The obtained sequence was submitted at EMBL-EBI gene bank under the accession number HE963840. A phylogenetic tree (Figure 2) was constructed based on the alignment sequences using MEGA 5.0 software. The phylogenetic tree of the isolated strain, *S. maltophilia* RSP-09 showed 100% 16S rRNA similarity with the strain reported from soil samples of Punjab region *S. maltophilia* strain 17A1. The interesting part of the present study is that none of the reported strains of *Stenotrophomonas* were found to produce lipase enzyme to the best of our knowledge.

Table 1. Biochemical Characterization of *S. maltophilia* RSP-09

Test	*S. maltophilia* RSP 09
Motility	+
Indole	-
Methyl red	-
Voges Proskauer	-
Citrate Utilization	-
Urease	+
Phenylalanine Deaminase	-
Nitrate Reduction	+
Catalase	+
H_2S	-
Starch hydrolysis	-
Gelatin hydrolysis	+
Colloidal Chitin hydrolysis	+
Casein hydrolysis	+
Carbohydrate	
Arabinose	-
Cellobiose	-
Dextrose	-
Fructose	+
Inositol	-
Maltose	+
Mannose	-
Mannitol	-
Rhamnose	-
Raffinose	-
Sucrose	-
Salicin	-
Xylose	-
Ribose	-

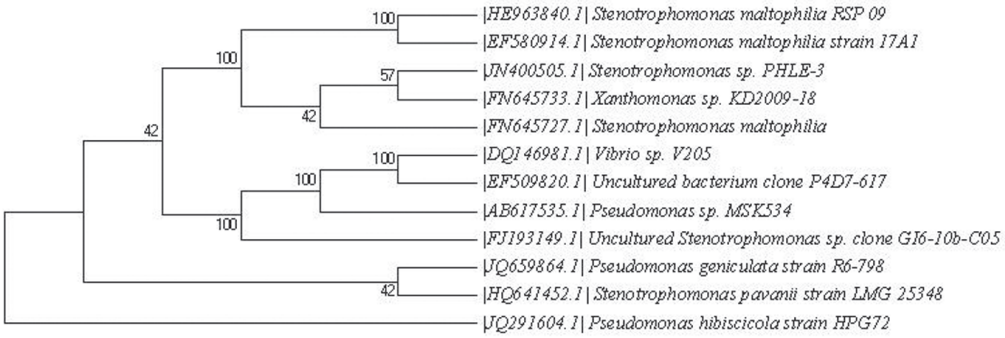

Fig 2. Phylogenetic tree of *Stenotrophomonas maltophilia* RSP-09

Characterization of Lipase

Medium development for lipase production by S. maltophilia RSP-09: *Stenotrophomonas* was rarely reported for lipase production though several *Pseudomonas* and *Xanthomonas* strains known for their lipase production extracellularly. *Stenotrophomonas maltophilia* is formerly classified under Xanthomonadaceae family and the strain is named as *Xanthomonas maltophilia*, however, this strain was separated based on the genotypic (DNA-ribosomal RNA and DNA-DNA hybridizations and G+C content) and phenotypic (comparative enzymology, type of ubiquinones, cellular fatty acid composition, growth characteristics, niche, etc) suggested that the *Pseudomonas maltophilia* should be included under the genus *Xanthomonas* as *Xanthomonas maltophilia.* Later Palleroni et al. (23) studied biochemical characterization of *Xanthomonas maltophilia* with other *Xanthomonas* species and suggested a new genus for *Xanthomonas maltophilia* as *Stenotrophomonas maltophilia* due to variations in the biochemical properties of this species with other *Xanthomonas* species (Table 2).

For commercialization of any microbial product, economic production and application spectra at industrial sector should be high. High yields of any enzyme are associated with efficient over expression of the corresponding genes which is influenced by the physiological and nutritional growth factors majorly or with the use of directed evolution. In view of the first report on lipase production by *S. maltophilia*, the isolate was investigated for its enzyme production titers under submerged fermentation using production medium at 37 ^{o}C and at 150 rpm. The enzyme production data suggested that lipase titers were noticed within 24 hours of incubation (minimum incubation time used for sampling for lipase estimation). The extracellular lipase production pattern was continued till 120 hours of incubation suggesting that the enzyme production by this *S.*

Table 2. Biochemical variation of *Stenotrophomonas maltophilia* with *Xanthomonas* species

Biochemical test	***Stenotrophomonas maltophilia***	***Xanthomonas species***
No. of flagella	>1	1
Fimbriae	+	-
Xanthomonadins	-	+
Xanthan gums	-	+
Asparagine as a C and N source	+	-
Nitrate reduction	+	-
Inhibition of growth by0.1% TPTC	-	+
Starch hydrolysis	-	variable
Chitin hydrolysis	+	-
Growth at 37°C	+	-
Oxidase reaction	+	-
Plant pathogenicity	-	+
Association with humaninfections and clinicalspecimens	+	-
Growth in the presence of0.01% methyl green,thionin, or lead acetate	+	-
Resistance to tetracycline,kanamycin, anderythromycin	+	-
Resistance to novobiocin	+	-

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maltophilia RSP-09 is growth associated and may be associated with supply of carbon and energy requirements for metabolic process of this strain. This could evidenced from observations when nutrient broth used as medium source, the production of lipase by this strain was not noticed throughout fermentation while, with the use of tributyrin as sole carbon source, extracellular lipase production was observed from the beginning of growth of the strain (data not shown) suggesting that the expression of lipase production in this isolate is inducible and fatty substrates are inducing factors. Further, growth of strain was vigorous in tributyrin supplemented medium compared to nutrient broth (data not shown) indicating the present isolate can be effectively grown in presence fatty substrates rather than carbohydrates as sole carbon source. Analysis of lipase production pattern denoted that lipase enzyme production is influenced by availability and type of carbon source and essential for metabolism towards growth of *S. maltophilia* RSP-09 (Fig. 3).

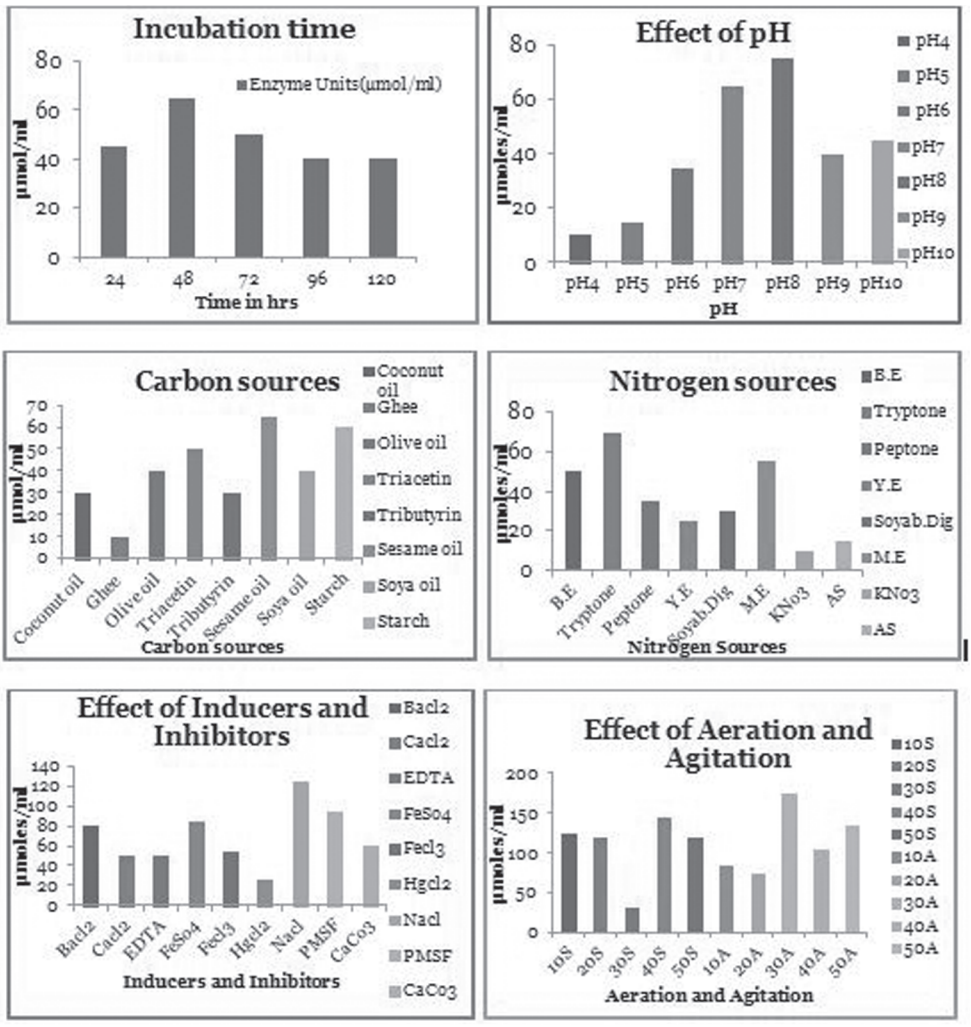

Fig 3. Lipase production optimization studies

Characterization of Lipase

Microbial growth under controlled environment is the key regulatory factor for biocatalyst production in high titers. pH is one of the major contributing factor involved in transport of certain nutrients across the cell membrane, metabolism of any microbial strain and influence the growth of the organism and subsequent product production. Evaluation of pH range for *S.maltophilia* RSP-09 growth associated lipase suggested that this enzyme production by this isolate is in wide pH range starting from acidic to basic pH however production titers are higher in alkaline pH (Fig. 3). Maximum amount of lipase production was observed at pH 8.0. whereas minimum amount of lipase enzyme production was observed at pH 4.0. With increase of pH up to 8.0 the enzyme production also increases gradually further increases in pH results in drastic fall of enzyme.

Carbon and nitrogen are most important constituents for structural and metabolic components of any biological entity. In general, any inducible enzyme production is associated with supplementation of complex carbon skeletons and high lipase production essentially require for lipidic carbon source though a few authors reported good yields in the absence of fats and oils (20). Different oil emulsions were tested for their efficacy to improve the lipase production. Among growth supplemented different carbon sources (coconut oil, tracetin, tributyrin, soya oil and sesame oil as well as starch), though all these supported growth associated lipase production however, differed in enzyme titer yields. It is interesting to know that lipase production also observed in starch supplemented conditions suggesting that this isolate also produces extracellular amylase enzyme and this glucose polymer supports the growth and metabolism of *S. maltophilia* RSP-09 (Fig. 3). This starch supported lipase production also not reported in literature. However, it is unclear at present that starch mediated amylase as well as lipase production and growth by this strain may be due to limited availability of carbon source during growth period in fermentation. Similarly, among tested nitrogen sources, tryptone supported maximum lipase production (Fig. 3). Further evaluation of the nitrogen source mediated enzyme production, inorganic nitrogen sources (potassium nitrate and ammonium sulfate) are the least supporters for lipase production by this organism compared to complex nitrogen sources (beef extract, tryptone, peptone, yeast extract and malt extract). Tryptone showed the maximum lipase production of 70 µmoles/ml after 48 h of incubation at pH 8.0 while ammonium sulfate supplementation resulted in production of <20 µmoles/ml (Fig 3). This complex nitrogen source based improved lipase production by this isolate may be due to the other nutrients which may be supporting the growth and metabolism of the *S. maltophilia* RSP-09.

***Effect of metal ions on the production of lipase enzyme*:** Different metal ions (Ba^{2+}, Ca^{2+}, Fe^{3+}, Hg^{2+}, Cu^{2+}, Na^{+}, EDTA and PMSF) were tested to improve the production of lipase enzyme (Fig 3). Among the metal ions used Na^{+} induced the lipase enzyme production where as Hg^{2+} inhibited. Sodium salts at a concentration of 0.1% improved the lipase production up to 125µmoles/ml where as mercury salts at a concentration of 0.1% inhibited the lipase enzyme production (Fig. 3).

Effect of aeration and agitation: The critical effect of aeration and agitation was studied in this experiment to improve the enzyme production. Two sets of experiment were performed in parallel i.e., one was at stationary and the other was at agitation speed of 150 rpm to study the effect of agitation. Effect of aeration was studied by varying the volume of fermented broth from 10ml to 50 ml in each condition. Maximum lipase production of 175µmoles/ml was observed for flask containing 30 ml of fermented broth at agitation speed of 150 rpm (Fig. 3).

***Partial purification and SDS-PAGE*:** Lipase enzyme was partially purified by ammonium sulphate precipitation method. The crude enzyme filtrate was subjected to four different fractions of

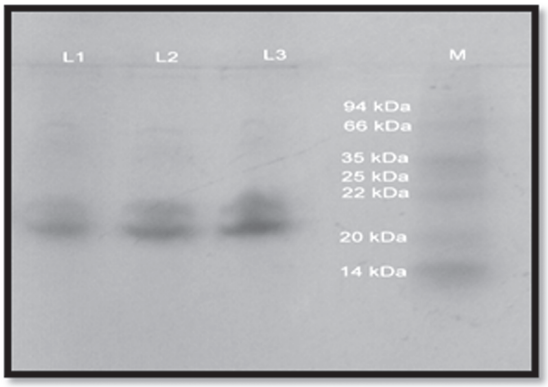

Fig 4. SDS-PAGE of lipase enzyme produced by *S. maltophilia* RSP-09

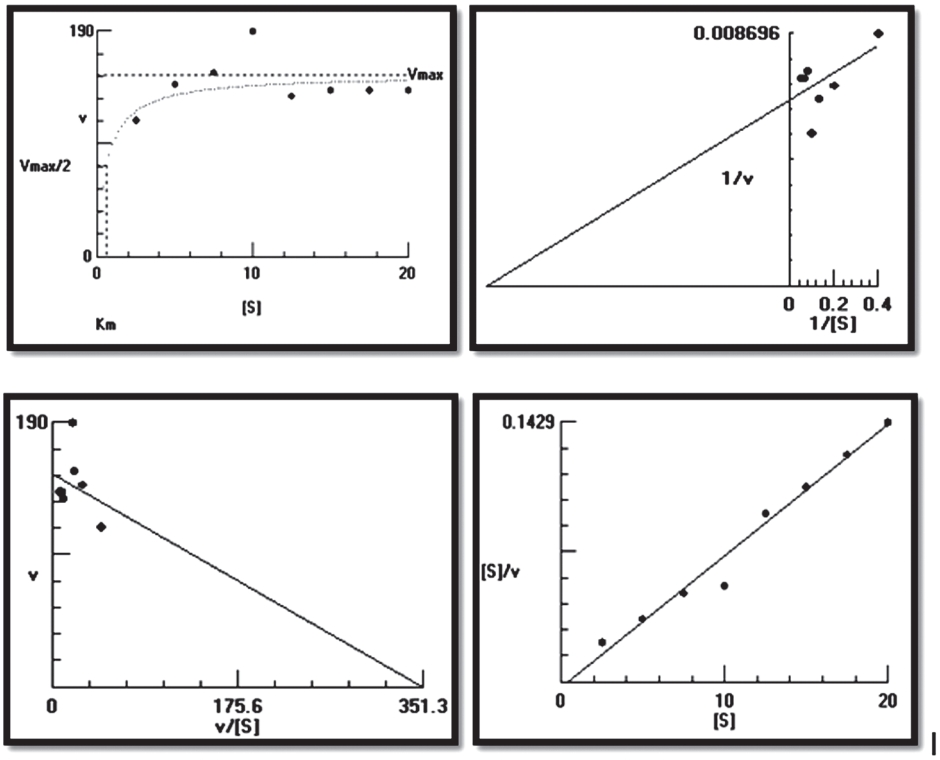

Fig 5. Kinetics plots for lipase produced by *S. maltophilia* RSP-09

ammonium sulphate precipitation at 30%, 50%, 80% and 100%. At each fraction the filtrate was centrifuged at 15,000 rpm for 15 min at 4 ^{O}C. The obtained pellets were dialyzed against 0.05M phosphate buffer of pH 7.2 to remove excess ammonium sulphate and analyzed for lipase. It was noticed that 80% fraction showed maximum enzyme activity. The fraction with maximum lipase

activity was kept for Native and SDS-PAGE to determine the molecular weight. Native PAGE studies revealed that the molecular weight of the produced lipase enzyme was approximately around 45 kDa (data not shown) where as SDS-PAGE studies revealed that the produced lipase has two polypeptide chains with almost equal molecular weight (Fig. 4).

***Kinetic studies and Activation energy*:** Effect of temperature and pH studies on the enzyme activity reveals that maximum lipase enzyme activity was observed at a temperature of 50 ^{o}C and at a pH of 6.0 respectively. Effect of substrate concentration studies showed that the maximum activity of lipase was observed for 10 % tributyrin emulsion concentration. The K_m and V_{max} for the produced lipase enzyme was determined by using Michaelis – Menten hyperbola (Fig. 5). The K_m value for the free enzyme, estimated from Lineweaver-Burk plot (Fig. 5), Eadie – Hofstee Plot (Fig. 5) and Hanes-Woolf plot (Fig. 5) were 0.727, 0.436 and 0.435 %, respectively with tributyrin as substrate. The Vmax values obtained from the three plots were 156.6, 153.2 and 138.5µmoles/ml, respectively. Energy of Activation (Ea) for lipase of *S. maltophilia* RSP-09 was 11.07 $kJmol^{-1}K^{-1}$ calculated with the help of Arrhenius plot. It was observed that at 50 ^{o}C the lipase had maximum catalysis in the conversion of tributyrin into free fatty acids by using Activation energy (Ea). Further increase in temperature, the enzyme showed reduced activity towards the conversion of the substrate into product. The observed lower activation energy indicated a good relationship between enzyme and the substrate.

Discussion

Lipase mediated biotransformations for synthesis of novel compounds is rapidly expanding. Lipases are characterized with unique catalytic properties to carry out reactions at the interface between aquatic and non-aquatic regions. This property provides these enzymes to utilize relatively a wide spectrum of compounds as substrates, high stability towards extremes of pH, temperature and organic solvents. One of the important characters of lipases is that they do not require cofactors for successful completion of reaction. In addition, the special properties like enantio-, chemo- and regioselectivity of lipases made them to play pivotal role in resolution of chiral drugs, fat modifications, synthesis of butter constituents and production of biofuel, personal care and flavours enhances. In view of their wide-ranging significance lipases remained a subject of enthusiasm and created tremendous interest among scientific and industrial communities however, these unique properties of lipases vary with source to source resulting one need to characterize each lipase for its specific properties before they are employed in industrial sectors and hence search for novel lipase producing microbes could be the continuous process.

Several bacterial strains were isolated using soil samples collected from oil industry localities around Hyderabad using the serial dilution method. In the present study lipase producing strains were identified by a tributyrin clearance zone around the colony (Fig. 1). Many researchers have isolated the lipase/esterase producing microbes from the different sources for example Yuan et al., (12) isolated cold adapted lipases strains from decaying seeds of *Ginkgo biloba* and Akanbi et al. (13) isolated lipase producing strains from Malaysian Hot Springs. Morphological, biochemical studies (Table 1) and molecular characterization studies revealed that the isolate belonged to Xanthomonadaceae family. This was further supported by similar studies performed by Padmapriya *et al.,* (14) and Charoenpanich et al. (15). The 16S rRNA gene sequencing analysis denoted that the sequence was 100% similar to the sequence of *Stenotrophomonas maltophilia* strain 17A1 and some similarity with other strains such as *Xanthomonas*, *Vibrio* and *Pseudomonas* species (Fig 2). A neighbor joining tree (Fig. 2) denoted that the isolate occupies a distinct Phylogenetic position within the radiation including representatives of the *Xanthomonadaceae* family. This highlights the novelty of the isolated strain and its significance of lipase production.

Maximum amount of lipase enzyme was produced at 48 h of incubation. Such data are in accordance with the literature data reported by Sirisha et al. (16). For the optimization of lipase production various parameters such as effect of pH, carbon and nitrogen sources, metal ions, and aeration and agitation studies were performed. Variation of pH studies revealed that maximum amount of lipase enzyme was produced when the pH of the production medium was 8.0 and up on gradual increase in pH from 4 to 8 the amount of lipase produced was increased up to pH 8 there after the production reduces drastically at pH 10 (Fig. 3). Similar results were observed by Sirisha et al., and Padmapriya et al., (14, 16). Optimization of carbon sources by different emulsions revealed that maximum amount of lipase enzyme was produced for sesame oil emulsion 65µmoles/ml and minimum production for ghee emulsion (Fig. 3). Gordillo et al. (17) observed that lipase production from *C. rugosa* for oleic acid. Lin et al., (18) produced an alkaline lipase from *P. pseudoalcaligenes* F-111 in a medium that contained both olive oil (0.4%) and Triton X-100 (0.2%). Essamri et al., (19) observed Rapeseed and corn oil were the most suitable substrates for cell growth and lipase production. Variation of nitrogen sources revealed that there was not much improvement of lipase enzyme which suggests that nitrogen sources do not show much effect on the optimization of lipase production (Fig. 3). Among the nitrogen sources used maximum lipase was obtained for tryptone.

Metal ions showed considerable effect on the production on lipase enzyme (Fig. 3). Among the different metal ions used sodium ions (Na^+) improved the lipase production up to 125µmoles/ml (doubled) where as mercury ions (Hg^{2+}) reduced the production rate to 25µmoles/ml (60 % reduction). Chartrain et al. (20) observed that an extracellular lipase of *Pseudomonas aeruginosa* MB5001 was strongly inhibited by $ZnSO_4$ (94% inhibition) but was stimulated by adding $CaCl_2$ (1.24-fold stimulation) and taurocholic acid (1.6-fold stimulation).

The molecular weight of the lipase was around 45 kDa and SDS PAGE studies revealed that the produced lipase enzyme has two polypeptide chains with almost identical molecular weights (Fig 4). Similar studies were performed by Matsumae and Shibatani (21) found that the molecular mass of *S. marcescens* SR418000 purified lipase was noticed to be 62 kDa. Arpigny and Jaeger (6) reported the molecular weight of lipase enzyme isolated from *Pseudomonas fluorescens* and *S. marcescens* was 50 and 65 kDa, respectively. Kinetic studies revealed that the produced lipase enzyme was highly active at temperature 50 ^oC and at 6.0 pH. Vmax and Km of the lipase enzyme was determined by using Lineweaver-Burk, Eadie – Hofstee and Hanes-Woolf plots suggest that the produced lipase had better affinity towards substrate (tributyrin) and better catalytic activity. The energy of activation (Ea) for the lipase enzyme produced by *S. maltophilia* was 11.07kJmol^{-1}K^{-1} which is the lowest reported energy of activation (Ea) for tributyrin substrate. Similar studies were reported by Ghori et al. (22) for *Bacillus* species using p-nitrophenyl laurate (pNPL) as substrate.

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Age- related Decrease in Rat Liver Catalase Expression is Associated with Change in its Promoter Methylation Pattern

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Abstract

Catalase is an antioxidant enzyme that catalyzes mainly the transformation of hydrogen peroxide, a reactive oxygen species, into water and oxygen. Reactive oxygen species cause oxidative stress that plays an important role in aging process. Results of the present study suggest that elevation of oxidative stress in liver during aging process is due to down regulation of catalase as a consequence of increase methylation of CpG island in its promoter region which spans from -268 to +52 with respect to translation initiation codon.

Keywords: Rat, catalase promoter, methylation, oxidative stress, aging.

Introduction

Catalase (EC 1.1.11.1) is an oxido-reductase enzyme and an important member of antioxidant defence system of aerobes. It plays a key role in protecting cells against reactive oxygen species (ROS) by breaking down hydrogen peroxide molecules those are generated due to dismutation of superoxide radicals by superoxide dismutase (7). Rat catalase gene is a single copy gene containing 13 exons and spans 33 kb. Promoter region of rat catalase is characterized by having eight CAAAT boxes, five GT boxes and lacks a TATA box. The gene has multiple initiation sites (18). It has been suggested that multiple complex formation of C/EBP-β, a transcriptional factor, which binds to catalase promoter, plays a crucial role in its transcription (24).

Alteration in DNA methylation is considered as a biomarker of aging (15) and has significant role in the development of age related diseases (27). It is reported that levels of DNA methylation in the promoter regions of genes are gradually augmented during aging (25). However, the age related methylation is tissue specific (26) and individual gene exhibits differential methylation patterns in different tissues during progression of age (12). Although, gene silencing by hypermethylation of promoter regions of genes in case of cancer is an established fact (10), its role in gene regulation of normal tissues under different physiological conditions needs attention.

Liver catalase has the dominant role in intracellular detoxification of hydrogen peroxide, and several studies have confirmed its down regulation with age (21,17,4,14). However, the biochemical basis of down regulation of catalase during aging is not well explained and warrants further study. Since regulation of expression of several genes are governed by methylation of their respective promoters (12), it is pertinent to explore the role of age on catalase expression by investigating the methylation status of its promoter. Information on the role of methylation in regulating the expression of catalase is not available except for a study by Min et al. (2010)

(16), where decreased expression of catalase is recorded due to hypermethylation of CpG island II in its promoter, as a consequence of augmentation of ROS in hepatocellular carcinoma cells. To understand the role of promoter methylation in catalase expression we compared methylation state of a specific region of catalase promoter (4421 to 4740) which spans from -268 to +52 with respect to translation initiation codon (+1 indicates A in the ATG translation start sites of catalase gene) along with its activity and translated products in the liver of young (30 days), adult (90 days) and old (700 days) male rats. In addition, lipid peroxidation level as an index of oxidative stress has been determined to establish a relationship between catalase expression and level of oxidative stress.

Materials and Methods

Animals and experimental design: Male Wistar rats (*Rattus norvegicus*), obtained from the National Institute of Nutrition (Hyderabad, India) were housed and breed in the animal room of the Department under standard conditions (12 h light and 12 h darkness cycle) as per guidelines of the Institutional Animal Ethics Committee (IAEC) regulated by the Committee for the purpose of Control and Supervision of experiments on Animals (CPCSEA), Government of India. In the present study young (30 days), adult (90 days) and old (700 days) rats were used. Rats were sacrificed by decapitation under ether anesthesia after recording their respective body weights. Liver was dissected out immediately, cleaned in cold normal saline (0.9 %, w/v), pat dried on filter paper and stored at -80 ^{0}C.

Lipid Peroxidation, catalase activity and catalase western blotting: A 20% (w/v) homogenate of stored liver samples (-80 ^{0}C) was prepared in 50 mM phosphate buffer, pH 7.4 with a Potter-Elvejhem type motor-driven glass teflon homogenizer. Supernatant obtained after centrifugation of the homogenate at 1000 x g for 15 min at 4^{0}C was used to determine the level of LPx by monitoring the formation of thiobarbituric acid reactive substances (TBA-RS) in the presence of 0.02% (w/v) butylated hydroxytoluene to suppress the endogenous peroxidation product (19). The concentration of TBA-RS was calculated from its extinction co-efficient, 1.56×10^5 M^{-1} cm^{-1} and was expressed as nmoles TBA-RS formed per mg protein. A part of the homogenate was treated with triton X-100 (final concentration 0.1 %, v/v) for 15 min and centrifuged at 10,000 x g for 15 min at 4 ^{0}C. The supernatant was used for catalase assay (4,1). One unit of CAT activity is defined as the amount of enzyme capable of catalyzing the decomposition of 1 µmol of H_2O_2 per minute. The activity was calculated by taking 43.6 M^{-1} cm^{-1}as the molar extinction co-efficient of H_2O_2 and finally expressed in microkat /mg protein. Protein concentration of samples was estimated according to the method of Bradford (2) by taking BSA as standard. Western blotting of catalase was performed with 1000 x g supernatant of liver homogenates prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 104 mM PMSF, 100 ìM E-64, 80 ìM aprotinin, 100 ìM leupeptin, 1% triton X-100 and 0.1% SDS to avoid protein degradation as described earlier. In brief, 25 mg of protein was resolved in 12% SDS-PAGE and transferred to PVDF membrane at 23 mA current for 1h. The membrane was blocked in 5% blocking solution for 1h at room temperature. The blot was then incubated with rabbit polyclonal anti-G3PDH (1:1000) and anti-Catalase (1:5000) for 1h at room temperature. The membrane was washed 3 times (each 10 minutes) with washing solution and subsequently incubated with HRP-conjugated anti-rabbit goat IgG (1:7500 for G3PDH and 1:10000 for catalase) for 1h at room temperature. Again the membrane was washed 3 times (each 10 minutes) with washing solution. After washing, specific immunoreactive proteins were detected in X-ray film with the help of ECL kit and band intensities were measured by densitometry.

***Methylation specific restriction enzyme digestion*:** 5 µg liver DNA isolated as per Sambrook et al. (22) was digested in 100 µl reaction volume with 10 units of restriction enzymes (MspI, HpaII; BamHI and HaeIII)

separately in respective buffers as supplied by manufacturer. Samples were incubated in shacking water bath at 37 °C for 16 h. The enzymes were inactivated according to manufacturer's protocol and the digests were stored at 4 °C. Two sets of primers were used in the present study. They have different binding sites in the catalase promoter region. Primer S1 (5'-ATCTCCCCAGCCTCTTCCCAT-3') binds catalase promoter which contains five CCGG sites, whereas, primer S2 (5'-CTCCTT CCAATCCTGTCCCTTCTAGA-3') binds catalase promoter which contains three CCGG sites. For both the forward primers, common reverse primer As (5'-AGATGAAGCAG TGGAAGGAGC-3') was used. Size of PCR products for S1 and S2 are 320 bp and 254 bp, respectively. PCR was performed in a Thermal cycler (My Cycler, BIO RAD) using above gene specific primers in a final reaction volume of 25 µl. Each PCR sample was prepared with positive and negative controls. PCR reaction mixtures were composed of 2 µl of restriction digests (containing 100 ng digested DNA), 2.5 µl of 10X buffer, 1.5 µl of 25 mM $MgCl_2$, 0.2 ìl of 10 mM dNTPs, 0.2 µl of 25 µM forward and reverse primers and 1.5 Units of True™ Start Taq DNA polymerase (Fermentas Life Science), and volume was adjusted with autoclaved milli Q water. PCR conditions were 5 min at 95 ºC, 1 step cycling, 30 s at 94 ºC, 30 s at 59 ºC (S1) and 52 ºC (S2), 60 s at 72 ºC for 32-35 cycles followed by 5 min final extension at 72 ºC.

Methylation specific primer PCR (MSP-PCR): To determine methylation status of catalase promoter by MSP-PCR, we have chosen a part of catalase promoter that spans from -184 to -34 as described in bioinformatic analyses (Materials and Methods section). One µg of liver DNA was treated with sodium bisulfite using the Epitect Bisulfite conversion kit. To analyze the methylation level of the catalase promoter, primers for MSP were designed using the meth primer software-The Li Lab (www.urogene.org/methprimer). In brief, 50 ng bisulfite-treated genomic DNA was amplified using both methylation-specific and unmethylation-specific primers. Methylation specific primer sequences were 5'-GAGTTTTAGTGGTTAATTAGGAGGC-3'(Forward) and 5'-GTAAAACAAA AAAACCG AACGAA-3' (reverse). Unmethylation specific primer sequences were 5'-GTTTTAGT GGTTAAT TAGGAGGTGG-3'(forward) and 5'-ACATAAAACAAAAAAACCAAACAAA-3' (reverse). PCR conditions were 95°C for 5 minutes; 35 cycles of 95°C for 30s, 54°C for 30s, 72°C for 60s and 7 minutes final extension at 72°C.

PCR products were analysed on 1.5% agarose gels using ethidium bromide staining and analyzed under gel documentation system (BIO RAD, Universal Hood II). Methylation and unmethylation status were determined by the band intensities of the PCR products.

Bioinformatics : Part of the rat catalase gene (Gene Bank AH 004967.1) spanning 320 bp (from 4421 to 4740) was analyzed for CpG island using the CpG Island Searcher programme http://cpgislands.usc.edu/cpg.aspx). The CpG island was defined as a DNA sequences of 200 bp with GC content more than 50%. CpG island of catalase gene has 65.7% GC content, and observed CpG /expected CpG ratio is 0.65. Moreover, the aforesaid region contains transcriptional as well as translational start points with several CCAAT sites. To recognize binding sites for transcription factor we used the TFSEARCH programme http://www.cbrc.jp/research/db/TFSEARCH .html).

Statistical analysis: Data was presented as mean ± standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range tests to find out the level of significance among the mean values. Band intensities of western blots and PCR products were quantified using computer assisted densitometry Image-Quant TL, Image Analysis Software v2003. Relative densities were determined as the ratio of catalase band/G3PDH (internal control) band.

```
4421 atctccccag cctcttccca  tcccggggtc  cacctcccgg  agcccactgc
4471 tcgccccacc ctccttccaa  tcctgtccct  tctagagttt  cagtggccaa
4521 tcaggaggcg gccgtcccga  gaggtggggg  gtggtgctga  ttggcagagc
4571 ctgaagtcac cactccagcg  ggcctgactg  acgcgattgc  ctaccccggg
4621 tggagaccgt gctcgtccgg  ccctcttgcc  tcacgttctg  cagctctgca
4671 gctccgcaat cctacaccat  ggcggacagc  cgggacccag  ccagcgacca
4721 gatgaagcag tggaaggagc
```

Fig. 1. Selected CpG island of promoter region of rat catalase gene from 4421 to 4740 (-268 to + 52) was considered for study of methylation pattern by restriction enzyme digestion and MSP PCR. Meth Primer program was used to select CpG island and primers for methylation specific PCR (MSP). All parameters were used as a default values.

Statistical significance between groups was considered at $p <$" 0.001 levels.

Results

Liver lipid peroxide level was found to be 35% higher in old rats in comparison to young rats (Table 1). Liver catalase activity showed a 35% decline in old rats in comparison to young ones. Relative level of its translated products decreased by 26% in liver of old rats than young ones (Table 1; Fig. 2). Ratios of PCR products of digested DNA samples by isoschizomers (MspI/ HpaII) of different age groups of rats are presented in Table 1. Presence and absence of PCR products in BamHI and HaeIII digested samples using S1 and S2 primers served as positive and negative controls for isoschizomer-digestion experiments, respectively (Fig. 3a,b). Ratios of band intensities of the PCR products of MspI to HpaII for primers S1 and S2 were 49% and 65% lower in old age in comparison to young rats, respectively (Table 1; Fig. 3c ,d). On the other hand, band intensity of MSP products of catalase promoter was 132% higher in the liver of 700 days rats than 30 days old ones (Table 1 and Fig. 3e).

Discussion

Oxidative stress is considered as a hallmark in the process of aging (8). The elevated level of LPx in liver tissues of 700 days old rats suggests the induction of oxidative stress in the old age. The decrease in catalase expression in 700 days old rats and hence a reduction in antioxidant activity could explain the elevation in lipid peroxides in aged rats. Our data are also in accordance with earlier reports which demonstrated reduce catalase activity along with elevated lipid peroxidtion in the liver of aging rats (6, 17, 21). The results of the present study advocate that decreased expression of catalase in aged rats may be associated with impairment of liver functions due to oxidative predominance as advocated by Schmukar (23).

Considering the important role of catalase in protecting liver from oxidative stress, we thought to explore the potential mechanism

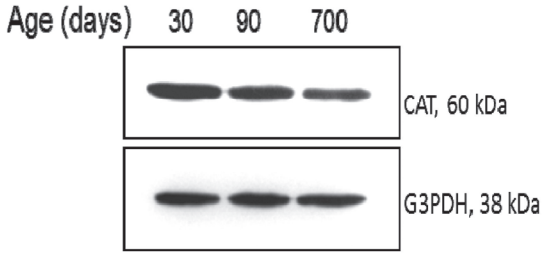

Fig. 2. Representative western blots of rat liver catalase of young (30 days), adult (90 days) and old (700 days) rats and G3PDH, used as internal control.

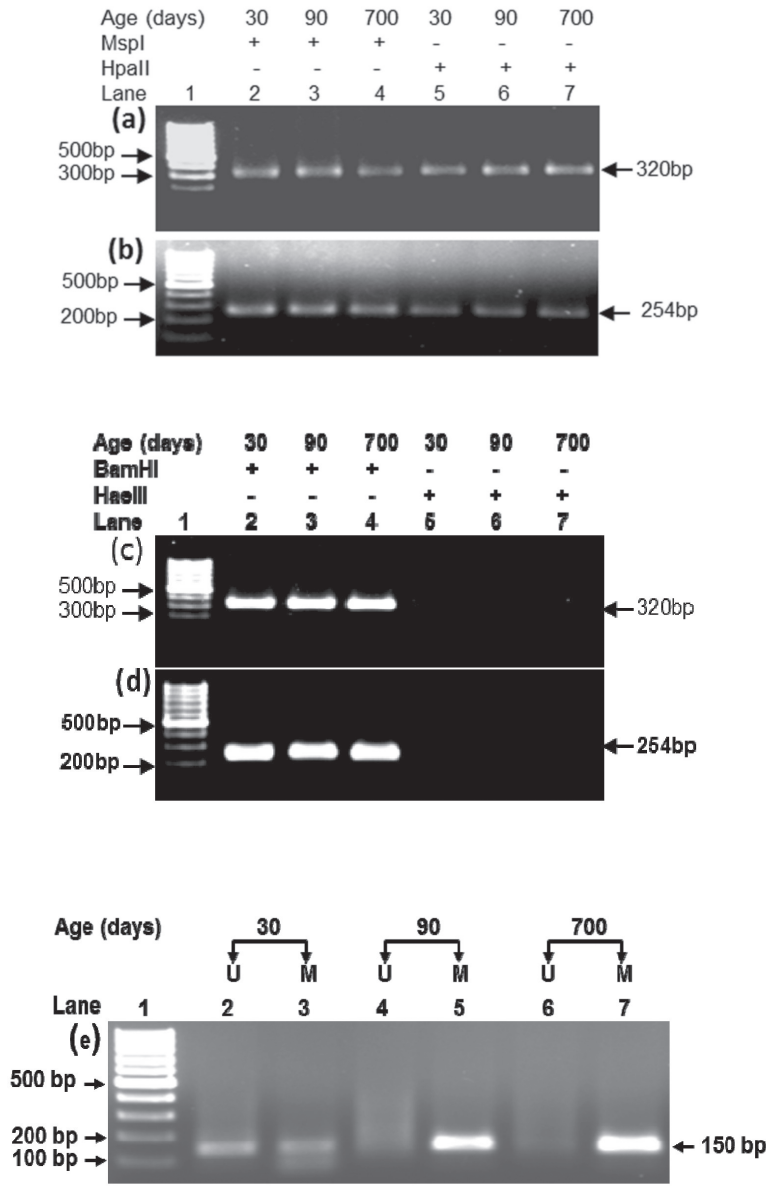

Fig. 3. Representative PCR products of promoter region of rat liver catalase gene of different age groups after digestion with MspI and HpaII restriction enzymes. DNA samples were taken from young (30 days), adult (90 days) and old (700 days) rats. (a) PCR product of MspI (lane 2 to 4) and HpaII (lane 5 to 7) with S1 and As primers (b) PCR products of MspI (lane 2 to 4) and HpaII (lane 5 to 7) with S2 and As primers. (c) PCR product of BamHI (lane 2 to 4) and HaeIII (Lane 5 to 7) with S1 and As (d) with S2 and As primers and loaded against 100 bp DNA ladder (lane 1). (e) Representative MSP PCR products in the liver of young (lane 2 to 3), adult (lane 4 to 5) and old rats (lane 6 to 7) and loaded against 100 bp DNA ladder (lane 1). U- represent unmethylated specific PCR product amplified with unmethylated specific primers, M- represent methylated specific PCR product amplified with methylated specific primers.

Methylation status of rat catalase promoter.

behind the down regulation of catalase with aging. Results of the present investigation suggest that differential methylation status of CpG islands of catalase promoter is possibly associated with the age dependent reduction in catalase expression. The region of catalase promoter as depicted in fig.1 (4421 to 4740) selected for the present study, is characterized by having eighteen CpG dinucleotides and several CCAAT sites. The region is also characterized by the presence of binding sites for several transcriptional factors such as AP1, SP1, C/EBPα, C/EBPβ and GATA1and GATA2 (18, 24). Bioinformatics analysis of the same region also supports the presence of the binding sites of the above transcription factors. To understand the role of methylation in the expression of catalase with respect to aging, we assessed methylation status of the same region of catalase promoter by analysing its sensitivity to methylation specific isoschizomers and methylation specific PCR. Conventionally it is believed that MspI cleaves

Table1. Age related changes in lipid peroxidation, catalase activity, translated products, ratio of band intensities of PCR products of digested DNA samples of MspI to HpaII and band intensities of the MSP products of the promoter region of catalase gene in the liver of young (30 days), adult (90days) and old (700 days) old male rats. For western blotting relative band intensities were determined as the ratio of catalase band/G3PDH (internal control) band. PCR products were quantified by densitometric analyses and expressed as arbitrary units (a.u.). Each data represent mean ± S.D. of five animals. Data having different superscripts in same row differs significantly (p<" 0.05) from each other.The ratio of band intensities of PCR products of digested DNA samples and MSP products of young rats was taken as 100%.

Parameters	**Age (days)**		
	30	**90**	**700**
Lipid peroxidation (nmol TBARS/mg protein)	3.67±0.19[a]	4.21±0.07[b]	4.98±0.10[c]
(%)	(100 ± 5 %)	(114 ± 1.9 %)	(135 ± 2.7 %)
Catalase activity (μkat/mg protein)	13.74±0.89[a]	10.85±0.51[b]	8.93 ±0.46[c]
(%)	(100 ± 6.74)	(87 ± 4.09)	(65 ± 3.34)
Catalase translated product (Integrated density value)	1.85±0.06[a]	1.61±0.05[b]	1.38±0.06[c]
(%)	(100 ± 3.24)	(87 ± 2.71)	(74 ± 3.21)
Ratio MspI/HpaII			
With S1 primer	1.45±0.90[a]	1.05±0.01[b]	0.74±0.12[c]
(%)	(100±6.0[a])	(72±0.9[b])	(51±16.0[c])
With S2 primer	1.59[a]±0.15	1.01±0.12[b]	0.56±0.07[c]
(%)	(100±9.0[a])	(64±1.02[b])	(35±12.0[c])
Catalase MSP product (Integrated density value)	52.30 ±4.97[a]	123.34±10.64[b]	172.10±13.75[c]
(%)	(100±9.50[a])	(136±11.74[b])	(232±18.54[c])

CCGG site when its cytosines are unmethylated (CCGG) or the internal cytosine is methylated ($C^{m5}CGG$) whereas, HpaII cleaves CCGG when its cytosines are unmethylated (28). Presence of PCR bands of DNA digested samples (MspI/ HpaII) of three age groups suggest that all five CCGG sites present in the specific region of catalase promoter are resistant to enzymatic cleavage. Decrease in intensities of PCR products of digested samples of old rats are 49% less in comparison to young animals. This indicates a change in methylation pattern of catalase promoter, which may be due to changing of sensitivity of CCGG sites to MspI and HpaII. It is possible that, in young age cytosines of CCGG are methylated in such a manner that they confer resistance to MspI. It is to mention here that MspI fails to cleave the sequence if cytosines are methylated at $^{m5}CCGG$ or $^{m5}C^{m5}CGG$ (13) or at 5'-$C^{m5}CGGCC$-3' (3, 11). On the other hand, HpaII fails to cleave the sequence if its cytosines are methylated ($C^{m5}CGG$ or $C^{m4}CGG$ or $^{m5}CCGG$ or $^{m4}CCGG$) or hydroxymethylated ($^{hm}5C^{m5}CGG$). Therefore, it is possible that changes in methylation pattern of cytosines of CCGG in old age make it more vulnerable to MspI and more resistant to HpaII, resulting in the decrease of the ratio. The same region of catalase promoter (-184 to -34) containing 11 CpG dinucleiotides is about 200% more methylated in liver of old rats in comparison to young ones as evident from methylation specific PCR (MSP) investigations. Observed inverse relationship between expression of catalase and methylation of its promoter in relation to age is in good confirmation with a earlier report, where a negative relation between the expression of liver glucokinsae and methylation of its promoter in old age was observed (9). It has been demonstrated that decrease catalase expression in hepatocellular carcinoma cell lines is attributed to diminished expression of Oct-I, a transcription factor, that regulates catalase expression (20). It is not out of context to mention that expression of catalase in hepatocellular carcinoma cells is reported to reduce as a result of hypermethylation of CpG island II in its promoter, as a consequence of augmentation of ROS (16).

In summary, we have demonstrated that, hypermethylation of hepatic catalase promoter is associated with the down regulation of catalase expression in aged rats and contributes to elevated oxidative stress in liver. However, the detailed mechanisms that link oxidative stress, down regulation of catalase expression, hypermethylation of catalase promoter and aging still merit further investigation

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NEWS ITEM

102nd meeting of Indian Science Congress

The 102nd meeting of Indian Science Congress was inaugurated by Honorable Prime Minister of India Sri. Narendra Modiji, held at Mumbai during 3-7 January, 2015 held at Mumbai. Hon. Prime Minister called for efforts to ensure that science, technology and innovation reach the poorest, the remotest and the most vulnerable person. He said that for a prosperous future for India, we need to put science, technology and innovation at the top of national priorities. In his address, the Prime Minister said more resilient agriculture, appropriate and affordable technologies for rural areas, improving healthcare, making clean technology affordable, and making India a leading manufacturing nation and a hub for knowledge and technology-intensive industries, were some of the key objectives before Indian scientists. The Prime Minister said a Nation's progress and its human development are linked to science and technology. He added that China's emergence as the second biggest global economy is in parallel to its rise to the second place in science and technology activities.

6th Indian Youth Science Congress

The 6th Indian Youth Science Congress held on January 19-21, 2015, at Acharya Nagarjuna University in collaboration with M.S. Swaminathan Research Foundation, was held at Guntur. The congress aims to provide a platform for young students and researchers to present their research work and discuss priority issues in enabling science and technology applications for human welfare. The young students will have an opportunity to interact with a galaxy of eminent scientists and policy experts in the field of Science and Technology.

OPPORTUNITIES

Post Doctoral Positin at Department of Biotechnology at Indian Institute of Technology (IIT), Roorke: The department of Biotechnology at Indian Institute of Technology (IIT) Roorke invites applications from outstanding and enthusiastic researchers for post dostoral positin under the mentor ship of Dr.Naveen K Navani (Associate Professor, Department of Biotechnology) focused on 'Development of Aptamer-Gold nanoparticles based system for detection of Toxic shock syndrome(TSS) Toxin-1'.

The review of applications will start on Jan 17, 2015 and continue until the position is filled. The department intends to fill the said position by Feb 12,2015. Interested candidates may send their resume with covering letter, list of publications, research plan to Head department of Biotechnology, IIT Roorke.

Postdoctoral Research Associate Positions at IISER Mohali: IISER Mohali invites applications at the level of Postdoctoral Research Associates. Interested applicants who have either a PhD degree or have submitted their PhD thesis can apply for these positions. This is a rolling advertisement and the applications will be reviewed from time to time. The salary for Postdoctoral Research Associates will be as per the MHRD norms. Applications should be sent by email to deanfaculty@iisermohali.ac.in with the subject clearly stating "Application for Postdoctoral position at IISER Mohali". The application packet should contain the following:A brief biodata with name, age, address, email and phone contact, subject of research interest, details of educational qualifications(after 10+2), PhD thesis title, thesis supervisor details, research highlights, and details of up to three prominent research publications, awards and distinctions (if any).

Postdoctoral Opportunities at NCBS - National centre for biological sciences: NCBS has a small but vibrant program for researchers who have a PhD degree. There are several funding modes for these postdoctoral programs. Applicants will be expected to identify a host laboratory and submit a preliminary application written in conjunction with the host faculty member.

Other Postdoc Postions

Postdoctoral positions available in computational and experimental neuroscience inStem - NCBS Single-molecule Imaging Laboratory. The inStem Stem Cell Biology LaboratoryinStem - NCBS Cardiomyopathy Laboratory

SCIENCTIFIC FINDINGS

Mini Ultrasound for Pain Relief

The miniature ultrasound device can soon be available to everyone suffering from arthritis or other pain. The novel device was created by George K. Lewis, the Cornell biomedical engineering graduate student, and is claimed to be the world's smallest ultrasound device. The device sends low-intensity ultrasound to the skin, which relives pain and may also be used to deliver various drugs. The mini ultrasound goes on the first clinical trial and may soon hit the markets.

New Device for Fertility Treatment

A device that mimics embryo experience in the body shows promise for infertility treatment. Scientists from University of Michigan developed a device that helps embryos feel more at home during IVF procedure by rocking them, which imitates embryo movements as they travel through fallopian tube to the womb. The study showed that embryos grown in a dynamic environment with the help of the new device not only resulted in more pregnancies, but also healthier embryos.

New Water-based Material that Can Replace Plastics

Scientists at the University of Tokyo managed to create a brand new material that is composed of 98 percent water. Researchers hope that their latest invention could one day serve as an alternative for petroleum-based plastics. The latest invention of the Japanese researchers is called Aqua Material and besides water, the new material is also composed of clay mineral that is sometimes used in cosmetics, sodium polyacrylate, which is a chemical that is used in diapers to absorb moisture and an altered form of a medical compound known as G3-binder.The strength of the new material is almost the same as the strength of silicon used in plastic surgery. In addition, the water-based invention can withstand temperature as high as 100 degrees Celsius. Researchers consider their latest invention can be used in medicine, for example in treating internal injuries. It is worth mentioning that information on the new material was published in the British science Journal Nature

Robot That Tests Visual Implants for Blind People

The latest invention of researchers from the California Institute of Technology (Caltech) is a remote-controlled robot that was developed to emulate the vision of a blind person who has a visual prosthesis implanted in the eye, for instance an artificial retina (aka retinal prosthesis). The latter is made of a silicon chip that has several electrodes, which directly stimulate the nerve cells found in the retina.

Biogel that Heals Brain Injuries

Recently it was addressed that in the next three years the field of medicine will benefit from the latest invention of U.S. researchers - an injectable "biogel" that can cure brain injuries of car crash victims as well as wounded soldiers. In 3 years scientists look forward to test their latest invention on patients.Biogel represents a mix of synthetic and natural chemicals. It stimulates neural stem cells which afterwards fix damaged nerves. Researchers decided to test the biogel on humans shortly after the studies on rats showed promising results. Scientists presented detailed information of their studies at the Military Research Forum that took place in Kansas City, US, and which has the goal of informing about the latest inventions in medicine that can help military troops. According to Dr Ning Zhang, the lead researcher in the biogel development team at Clemson University in South Carolina, there has been an increasing number of brain injuries among soldiers and the biogel could help them all of them recover. You can find more in-

ventions (including those from the field of medicine) here at www.InfoNIAC.com - check the links at the bottom of the story. But, biogel can also be used to help patients with head injuries that occurred as a result of car accidents and falls. The biogel is injected into the patient's wound site in order to direct the reaction of neural stem cells. It is worth mentioning that stem cells are able to produce various types of tissue, thus, in the brain, they can generate nerve cells. Chemicals contained in the biogel make it possible for neural stem cells to restore normal brain tissue in the head wound.

Fungi Makes Plastic Waste a Tasty Treat

The Austria-based Livin Studio together with Utrecht University have developed the Fungi Mutarium, a kind of incubator designed to cultivate edible fungi that digests plastic as it grows. The two main fungi used are already eaten in households around the world: *Pleurotus ostreatus*, or Oyster Mushroom, and Schizophyllum Commune, or Split Gill, which is common Asia, Africa and Mexico. "Both fungi show characteristics to digest waste material while remaining edible biomass," Livin Studio founder Katharina Unger told Dezeen. But in this case, the mushrooms are grown in edible egg-sized pods made from agar, derived from seaweed. A culture of mycelium the fibrous part of the mushroom is added to the pod along with some plastic. As the culture of mycelium grow, it digests the waste material and fills up the little egg-sized pod.

Smart Clothes That Keep You Healthy

Scientists develop new age clothes using fabrics with tiny electronic devices and using other latest inventions in medicine incorporated into clothes to monitor the health of its wearers. Now you can make use of some of these intelligent garments that are able to fight unpleasant body odor, warn a person about the early signs of breast cancer, control blood pressure and even fight off viruses and bacteria.

SCIENTIFIC NEWS

1. Scientists find Promising New Antibiotics The bacterium, *Eleftheria terrae*, yielded an entirely new sort of antibiotic

Using a novel technique to culture soil bacteria that previously could not be grown in the laboratory, a team of U.S. scientists led by Dr. Kim Lewis, director of the Antimicrobial Discovery Center at the Northeastern University in the U.S., and colleagues used an 'isolation chip' (iChip) developed at the university to culture previously uncultivable soil bacteria. With the iChip, the scientists could grow 10,000 bacterial strains. The extract from one such bacterium, provisionally named *Eleftheria terrae*, yielded an entirely new sort of antibiotic, teixobactin. The research, published this week in *Nature*, comes at a time when there is growing alarm both at the spread of antibiotic-resistant microbes and the failure to find new classes of antibiotics in recent decades.

2. IISc: Repurposing Existing Drugs to Fight TB

Small changes to the molecules can turn them into effective TB drugs a proof-of-concept study has successfully identified two small molecules- imipramine and norclomipramine that can arrest the growth of TB bacteria and hence have the potential to be used as anti-TB drugs once the chemical properties are altered to make it more effective. Prof. V. Nagaraja of the Department of Microbiology and Cell Biology, Indian Institute of Science (IISc), Bengaluru led the team that identified the inhibitors. The two small molecules work by targeting the Topoisomerase I enzyme of the TB bacteria. The two inhibitors prevent the enzyme from functioning. The inhibition of the enzyme arrests the growth/division of the bacteria and eventually causes death. The results of the study were published recently in the Journal Antimicrobial Agents and Chemotherapy.

3. Using Silk to Measure Blood Sugar

Achira Labs, A Bengaluru-based start-up has designed an eco-friendly blood glucose monitoring strip made out of silk. It will be made

by wavers with traditional weaving skills in five cities in Karnataka and Tamil Nadu. While conventional strips are made of plastic embedded with electrodes that allow the machine to measure the glucose in the blood, the silk strips have electrodes woven into the fabric. Grand Challenges Canada and Indian Council of Medical Research (ICMR) funded the research for the product, which the company has now patented.

4. Taj: The Pollutants Causing Discolouration Identified

Finally, the specific pollutants in the air that are responsible for the discolouration of the white marble of Taj Mahal have been identified. Particulate carbon and fine dust particles that are deposited on the marble are responsible for its browning. The results from a study were published a few days ago in the Journal Environmental Science & Technology. "There is one group of organic carbon which absorbs light in the blue region of the spectrum and this is called brown carbon. Discolouration is because of what is happening to reflectance, and reflectance is in turn influenced by these particles," said Prof. S.N. Tripathi from the Department of Civil Engineering and Centre for Environmental Science and Engineering, Indian Institute of Technology (IIT), Kanpur. He is one of the authors of the paper.

5. Insight into Origins of Deadly H7N9 strain

Changes in a flu virus that has plagued Chinese poultry farms for decades helped create the avian H7N9 influenza A virus that has sickened more than 375 people since 2013, say researchers.

6. Red Meat Diet Linked to Cancer Risk in Mice

A red meat sugar called Neu5Gc, naturally found in mammals, but not in humans, when fed to mice engineered to be deficient in the sugar (like humans) significantly promoted spontaneous cancers.

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Collaboration with JNTUH, India: Alliance is having collaboration with **Jawaharlal Nehru Technological University, Hyderabad (JNTUH)**, which is a premier institution with academic and research–oriented programs, offered through the constituent and affiliated colleges. Alliance's syllabi, academic regulations and course structure are **approved by the JNTUH. JNTUH awards the degrees after fulfilling the degree requirements.**

Collaboration with University of the Pacific, USA: University of the Pacific, ranks in the top 100 among the 3000 national universities in the United States. Alliance has entered into research collaboration with Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific.

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