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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

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

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Selection of Reference Genes for qRT-PCR Normalization to study *Hif1α* and *Hif2α* Expression in Hypobaric Hypoxia Susceptible and Tolerant rats lung

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Abstract

Acute hypobaric hypoxia may damage brain and lung tissues due to the development of disorders such as High Altitude Cerebral Edema (HACE) and High Altitude Pulmonary Edema (HAPE). Rats, like humans, exhibit susceptibility and tolerance in the same population to such extreme conditions. We selected reference genes to normalize the qRT-PCR study in order to evaluate whether a change in expression of *Hif1α* and *Hif2α* occurs in lungs of such rats exposed to acute hypobaric hypoxia.The hypobaric hypoxia susceptible (HHS) and tolerant (HHT) Sprague-Dawley rat groups, formed on the basis of their gasping time, were exposed to a simulated altitude of 9144 m at 24°C in a decompression chamber for a short duration of one hour. A set of reference genes including *Gapdh*, *Actb*, *Rpl11*, *Rpl10a*, *Rps15* and *Ppia* was examined for normalization in qRT-PCR study to analyze expression of target genes *Hif1α* and *Hif2α* in the lungs of these groups as compared to the normoxic control. *Rpl11, Actb* and *Rps15* genes in combination represented the most suitable reference genes based upon GeNorm, NormFinder and BestKeeper analyses. Expression of *Hif1α* and *Hif2α* genes was reduced in both HHS and HHT rat lungs. However, enhanced protein expression of HIF-1 in HHS group, and weak expression of HIF-2 protein in both groups was recorded. Our findings suggest that HIF-1 may play a significant role in mediating early responses towards acute hypobaric hypoxia in lung samples of HHS as well as HHT rats, with its profound protein expression in the former.

Key Words: Hypobaric hypoxia, Gene expression , Tolerance to high altitude, Acute hypoxia

Introduction

Hypobaric hypoxia is characterized by oxygen deficiency in the body due to a progressive decline in atmospheric pressure with ascending altitude. It may lead to acute altitude illness that begins after a few hours to days of continued exposure to hypoxia and is categorized into Acute Mountain Sickness (AMS), High Altitude Cerebral Edema (HACE), and High Altitude Pulmonary Edema (HAPE). AMS begins with headache and progresses with neurological dysfunction that may cause brain swelling as a consequence of fluid accumulation due to increased blood brain barrier permeability and intra-cranial pressure. In severe condition, it leads to HACE, which may cause death due to brain herniation (1). Onset of HAPE has been postulated to result from different events like pulmonary capillary stress failure depicting the hydrostatic mechanism (2), uneven vasoconstriction leading to regional capillary over

perfusion (3), and calcium influx with closed voltage gated potassium channels (4). A further increase in lung damage advances to death if proper medication and treatment is not provided on time.

Some populations and, in fact, some individuals within a population having augmented arterial systolic pressure show more predisposition towards development of pulmonary edema than others depicting variable response to hypoxia adaptation (5). Females appear to be more resistant towards it suggesting a natural gender bias (6). Some genome wide association and proteome based studies have been done on high altitude adaptation on blood samples obtained from different populations (7, 8). Although, some polymorphisms have been found associated with susceptible and resistant hypobaric hypoxia in populations (9), however, the genetic and molecular pathways of disease progression in hypobaric hypoxia remain unexplored.

It is well known that HIF1 transcription factor binds to the hypoxia responsive element (HRE) and regulates the expression of different genes involved in angiogenesis, glycolysis and NO production contributing to various adaptation mechanisms operating during exposure to hypoxia, HIF2 regulates expression of genes such as *Epo*, *Oct4* and those involved in cell cycle progression, NOTCH signaling and inhibition of NO production (10). HIF2 has recently been linked to long term adaptation in Tibetans to high altitudes (11). Owing to the involvement of HIF1a and HIF2a subunits in these adaptive responses to hypobaric hypoxia, we hypothesized that these master regulators might be differentially expressed in rats, which are either susceptible or tolerant to extreme hypobaric hypoxia. For that purpose, rats were screened and segregated into hypobaric hypoxia susceptible and tolerant rats on the basis of their gasping time by exposing them to a simulated altitude of 9754 m (12). After a week of acclimatization, these two groups were later subjected to a simulated altitude of 9144m for one hour to study their response to acute hypobaric hypoxia stress. The gene and protein expression of HIF-1 and HIF-2 were examined in lung samples obtained from these groups.

Quantitative Real Time Reverse Transcription Polymerase Chain Reaction qRT-PCR study was used to measure the mRNA expression of target genes- *Hif1*α and *Hif2*α. It is a highly efficient technique to study expression of individual genes and for validating high throughput gene expression profiling studies. However, before undertaking any qRT-PCR experiments, it is a prerequisite to select optimized normalization factors or reference genes that could serve as ideal controls for evaluating the target gene expression. This is done in order to minimize non-biological variations in a comparative gene expression analysis (13).

Six reference genes namely *Gapdh*, *Actb*, *Rpl10a*, *Rpl11*, *Ppia* and *Rps15* were considered to validate and select suitable reference genes for normalization in our qRT-PCR study. Most studies use *Gapdh* and *Actb* as reference genes or so-called house-keeping genes. However, their use is limited owing to their differential mRNA expression under different experimental conditions. Three online softwares available for normalizing the gene expression were employed in the study: GeNorm (14), NormFinder (15) and BestKeeper (16). The outputs of these softwares were then compared and used to evaluate transcript expression of *Hif1*α and *Hif2*α in the rats kept under normoxic condition as control (Con), hypobaric hypoxia susceptible (HHS) and hypobaric hypoxia tolerant (HHT) groups. In order to verify whether the mRNA expression correlates with the protein expression, western blotting for HIF-1 and HIF-2 proteins was also carried out with Con, HHS and HHT lung samples. Our study is an important effort in the direction of understanding response to hypobaric hypoxia stress and subsequently devising strategies to develop more effective medicines and therapy to benefit travelers, visitors, sport persons and soldiers exposed to acute hypobaric hypoxia during their ascent to high altitude.

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Methodology

Experimental Animal : Sprague Dawley male albino rats of weight 185 ± 10 g were maintained on a daily standard pelletized diet and sterile water with temperature 24±2°C maintained inside the animal house facility of DIPAS, DRDO, New Delhi. The rearing conditions of animals and all the below mentioned protocols of acute hypobaric hypoxia exposure were approved by Institutes Animal Ethical Committee of Defence Institute of Physiology and Allied Sciences (DIPAS), DRDO and all the guidelines were in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Group Segregation into Hypobaric Hypoxia Susceptible and Tolerant Rats : The rats were selected on random basis and exposed to acute hypobaric hypoxia by gradually decreasing the atmospheric pressure to obtain a simulated altitude of 9,754 m and barometric pressure of 205.8 mm Hg at 32°C in an animal decompression chamber (Decibel Instruments, Delhi, India) attached to a mercury barometer. The desired altitude equivalent was achieved by incrementing simulated altitude at a rate of 3,000 ft per min (914 m/min). The relative humidity was maintained at 40-50% with airflow around 2 L/min in the chamber. The time taken for the appearance of first sign of gasping was recorded as gasping time using an electronic stopwatch. Rats were segregated into two groups HHS (gasping time less than 5 minutes) and HHT (gasping time greater than 45 minutes) on the basis of gasping time. A third group of normoxia labelled as Con (rats never exposed to hypoxic stress) was kept as control and maintained at standard conditions as mentioned above. All the three groups were kept for acclimatization under normal conditions at 24±2°C for a week.

Final Hypobaric Hypoxia Exposure and Tissue Collection: All the three groups (n=6 in each case) were then exposed to a simulated altitude of 9,144 m and atmospheric pressure of 225.6 mm Hg maintained inside a decompression chamber at 24°C for 1 hour. Same conditions of relative humidity and airflow were maintained as stated above. The rats were anaesthetized using ketamine (80 mg kg^{-1} body weight, *i.p.*) and xylazine (20 mg kg^{-1} body weight, *i.p.*) and sacrificed to collect lung tissue for the qRT-PCR expression study. These samples were stored at -80°C.

RNA Extraction and cDNA Synthesis: Total RNA was extracted using TriReagent (MRC Inc.) from lung samples of all the three groups. These RNA samples were subjected to quality analysis and concentration estimation using Nanodrop and samples having 260/280 absorbance ratio between 1.9 and 2.0 were treated with DNase1 (Fermentas) to remove any traces of genomic DNA. RNA was then purified using Chloroform: Isopronanol precipitation and dissolved in nuclease free DEPC treated water after ethanol wash. These samples were again tested for RNA integrity using UV-Vis spectrophotometer. Good quality RNA samples were subjected to first strand cDNA synthesis using ThermoScientific Revert Aid First strand cDNA synthesis Kit.

Primer Designing and qRT-PCR Optimization: The cDNA sequences of *Gapdh*, *Actb*, *Rpl11*, *Rpl10a*, *Rps15*, *Ppia, Hif1*α and *Hif2*α were downloaded from NCBI Entrez Nucleotide database. These sequences were used as templates for primer designing using PrimerBLAST database of NCBI (17) using default parameters, except that the length of the amplicon was kept between 70 to 150 bp taking mRNA *RefSeq* database of *Rattus norvegicus* as reference. Oligo*Calc* software (18) was used online to check any non-specific template binding, hairpin loop and dimer formation in designed primers (Table1).

The cDNA samples were used as qRT-PCR templates after primer optimization and optimum template dilution using RT-PCR and gel electrophoresis in order to standardize the conditions for amplification of the desired reference and target genes. Quantitative RT-PCR was carried out using Syber Green dye for labelling any double stranded amplicon in the reaction mixture in a 96 well Real Time thermal

Table I: Details of the candidate reference genes and the target genes studied.

Gene Symbol	Gene Name	NCBI Accession No.	Forward Primer (5'-3')	Reverse Primer (5'-3')	Tm (°C)	Product Size (bp)
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	NM_017008.4	AGGCTGGGGCTCACCTGAAG	GCAGTTGGTGGTGCAGGATGC	57±0.2	149
Rps15	Ribosomal protein S15	NM_017151.2	CCAGGTGGAGATCAAACCCG	TGCTTCACAGGCTTGTAGGT	54±0.3	75
ActB	Actin, beta	NM_031144.3	CAGGGTGTGATGGTGGGTATGG	AGTTGGTGACAATGCCGTGTTC	55±0.2	115
PPIA	Peptidylprolyl isomerase A (cyclophilin A)	NM_017101.1	GTCAACCCCACCGTGTTCTTC	ATCCTTTCTCCCCAGTGCTCAG	55±1.6	133
Rpl11	Ribosomal protein L11	NM_001025739.1	GTCCTTTGGCATCCGGAGAA	GTACTCTCGCACCTTCAGGC	54±1.3	103
Rpl10a	Ribosomal protein L10A	NM_031065.1	CCGCAGCCATGAGCAGCAAA	CTGCAGCTCCACCGTCTCCA	59±0.4	113
Hif1á	Hypoxia inducible factor 1 subunit alpha	NM_024359.1	TCCTGCACTGAATCAAGAGGTTGC	ACTGGGACTGTTAGGCTCAGGTG	58±0.3	151
Hif2á	Hypoxia inducible factor 2 alpha	NM_023090.1	GTCGGCCACCTGGAAGGTCT	ATGAGGCAGGACAGCAGGGG	57±0.7	111

cycler (Agilent Technology, MX3000P). The initial denaturation was performed at 95°C for 3 min followed by 40 cycles of three step RT-PCR: 95°C for 20 sec, annealing temperature of 55°C for 20 sec and extension at 72°C for 15 sec in each cycle. Efficiency of each primer pair was checked by serial dilutions to obtain a standard curve. Reactions were carried out in triplicates including those for standard curve and No Template Controls (NTC) in each run of the experiment. The melting curve analysis was simultaneously done to check and rule out the formation of non-specific products and primer dimers.

qRT-PCR Data Analysis: qRT-PCR results were analysed using these softwares: GeNorm (14) , NormFinder (15), and BestKeeper (16).

GeNorm analysis**:** GeNorm calculates the pairwise variation ratios of Cq values of one candidate reference gene with that of other, which represents the standard deviation obtained from the log transformed expression ratios. A reference stability value called 'M value' is calculated as average pairwise variation of one candidate reference gene from all others. All the six reference genes were then studied in the present study according to their M values.

NormFinder analysis**:** The log transformed values of ΔCq values were calculated from the Cq values obtained for all six genes expressed in Con, HHS and HHT rats, and then subsequently, used as an input to NormFinder software. The software was used to calculate the expression stability values for all the genes along with the inter- and intra-group variations.

BestKeeper analysis: Raw Cq values were taken as direct input by BestKeeper software for geometric mean calculation following the pair-wise correlation analysis of all candidate normalization gene pairs. The software also carried out regression analysis and the genes having the standard deviation >1 from the calculated BestKeeper Index were excluded leaving behind the suitable reference genes.

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Target Gene Expression Analysis: The reference genes found to be stable in terms of expression in all the three software were taken for further analysis. Their geometric mean was calculated and Livak method (19) was used subsequently to analyze the differential expression pattern of *Hif1*α and *Hif2*α in HHS and HHT samples as compared to Con sample utilizing value $2^{\Delta\Delta Cq}$ as approximate fold change or the relative quantification value. The Cq denotes the amplification cycle or threshold at which the real time curve crosses baseline and the superscript ΔCq (Con-HHS (or HHT) is the deviation of ΔCq value of sample (either from group HHS or HHT) versus Cq value of Control.

Protein Expression Analysis: The protein expression of the target genes *Hif1*α and *Hif2*α in the lung tissue samples from Con, HHS and HHT groups was determined by developing western blots. An ice cold buffer A (0.5 M sucrose, 10 mM HEPES, pH 7.9, 10 mMKCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM phenyl-methyl sulphonyl fluoride) containing protease inhibitors cocktail (Sigma) was added four times the amount of tissue sample to homogenize the lung tissue. After 15 minutes of incubation, NP-40 (Nonyl phenoxypoly-ethoxylethanol) was added to the homogenate to make upto 0.6% of the total volume of the solution and centrifuged at 2000 g for 10 min at 4°C. For nuclear fraction, the pellet obtained after removing the supernatant was re-dissolved in equal volumes of ice cold buffer B (20 mM HEPES, pH 7.9, 0.3 mM NaCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenyl-methyl sulphonyl fluoride) containing the same protease inhibitors cocktail as mentioned above. This solution was incubated on ice for about half an hour and centrifuged at 20,000 g for 15 min at 4°C. The supernatant aliquots containing nuclear fraction were stored at -80°C for immunoblotting and protein estimation. Lowry's method was used to estimate concentration of total protein in each sample aliquot. The proteins in each sample were separated using SDS-PAGE by loading 50 μg of each sample. These gels were electro-blotted on to a nitro-cellulose membrane (Millipore, USA). This nitro-cellulose membrane bearing protein bands was blocked with 3% BSA for 2 hours and then washed with Tris buffer saline with Tween 20 (0.1%) to decrease non-specificity. The blocked membranes were probed with their respective primary monoclonal antibody of HIF-1 obtained from mouse (1:1000 dilution) and polyclonal antibody of HIF-2 from rabbit in the ratio of 1:500 dilution (Santacruz, CA, USA) for 3 hours. After washing with TBST, the membranes were incubated with secondary antimouse and antirabbit-IgG-HRP conjugate (each with 1:25,000 dilution) sequentially for 2 hours. These membranes were then incubated with chemiluminescent substrate (Sigma) to visualize the specific protein bands. Bands were developed on X-ray films (Kodak, USA) in dark.

Results

qRT-PCR Expression Analysis in Control, Susceptible and Tolerant Groups: For performing qRT-PCR, cDNAs from the three groups, *i.e.*, Con, HHS and HHT were synthesized as described in methodology. The PCR efficiency predicted on the basis of the slope of standard curves in case of all the primers was found to be in the range 90-95%, which is within the acceptable limits. Single peak obtained in the melting curve analysis in each case and Cq values above 35 cycles observed in the no template control (NTC) indicated that there was no contamination in the reactions and PCR products were primer specific.

qRT-PCR Data Analysis for Optimum Reference Gene Selection: Three softwares that work on different algorithms and principles were employed to determine the most stable genes suitable for normalization in qRT-PCR. These softwares have been used in recent studies (20, 21). GeNorm calculates the log transformed values itself to calculate stability value, while NormFinder uses the log transformed Cq values or ΔCq values (base 2) and converts it into natural base e log values to calculate intra and inter group variations along

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with a different stability values. BestKeeper directly takes Cq value irrespective of group to which they belong and calculates the standard deviation with respect to BestKeeper index along with pairwise correlation coefficient and power fold change indicating the difference in the expression of the gene under test condition as compared to the control.

Three sets of data were used for comparison of gene expression during hypobaric hypoxia (no treatment vs hypoxia treatment) and included: Con vs HHS, Con vs HHT, and Con vs combined (HHS and HHT). The GeNorm analysis indicated that in all the three groups, the order of the 'M' stability value and pattern of graph remains similar that is *Gapdh*> *Ppia*> *Rpl10a*> *Rps15*>*Actb*>*Rpl11* (Figure 1). This finding indicates that *Rpl11* and *Actb* with M values less than 0.6 are highly stable genes whose expression does not vary much as compared to other genes under study. This observation is also evident while comparing a normalization factor from the V_n/V_{n+1} values that remained less than 0.15 for the 2 or 3 most stable targets. *Gapdh* having highest 'M' value (always above 0.9) in each case seemed to be the most unsuitable candidate having highest variation in terms of expression. The analysis suggested that the geometric mean of reference targets *Rpl11* and *Gapdh* will serve well as optimum factor for normalization.

When NormFinder algorithm was used, the order of stability value remained *Gapdh*>*Ppia*>*Rps15*>*Actb*>*Rpl10a*>*Rpl11* for Con vs HHS, *Gapdh*>*Ppia*>*Rpl10a*>*Rps15*>*Actb*>*Rpl11* for Con vs HHT, and Rpl10a>*Gapdh*>*Ppia*>*Rps15*>*Actb*>*Rpl11* for Con vs both (HHS and HHT). *Rpl11* and *Actb* with the lower stability values than others were found to be the most suitable reference gene in Con vs HHS, Con vs HHT and Con vs both (HHS and HHT) (Figure 2). The resultant inter-and intra-group variation in combined comparison (Con vs HHS and HHT) calculated from the log transformed values of expression revealed that *Rps15* and *Rpl11* had least intra-group variation as visible through error bars in Con vs HHS, Con vs HHT and Con vs both (HHS and HHT) groups in the graph, whereas *Rpl11* and *Actb* were found to have least inter-group variation in the same group (Figure 3). Thus, it was concluded from the present results that *Rpl11* and *Actb* were most stable reference genes having minimum stability values and variation within and across all sample groups.

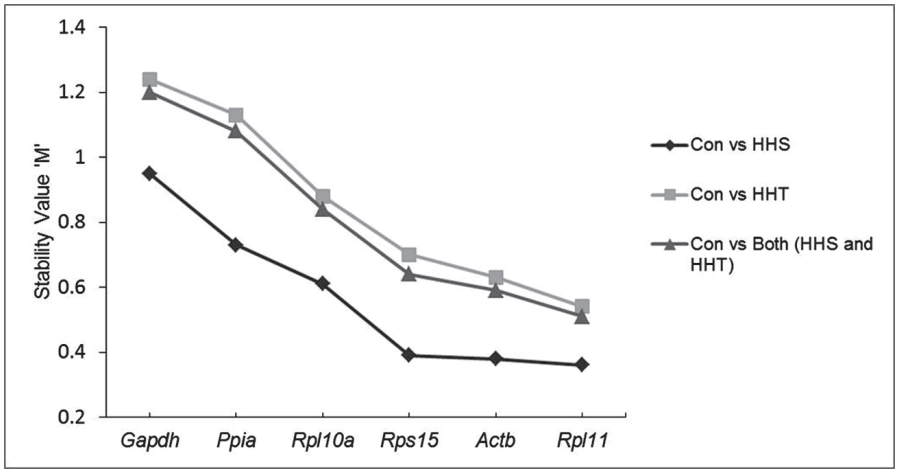

Fig. 1. Graph representing curves obtained by plotting stability values 'M' of GeNorm for Con vs HHS, Con vs HHT and Con vs both (HHS and HHT) group. Most suitable reference gene *Rpl11* has least M value, while *Gapdh* is least suitable reference gene with highest M value in all three comparisons

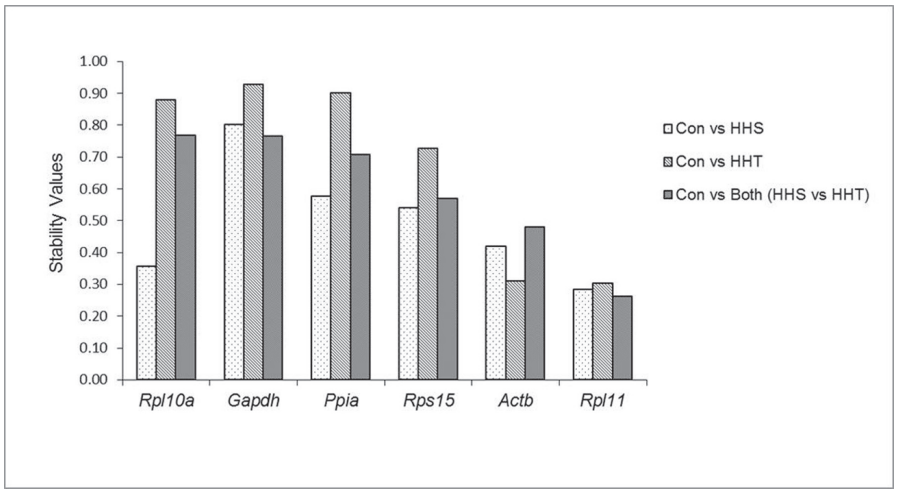

Fig. 2. Plot obtained by plotting stability values calculated by NormFinder software for Con vs HHS, Con vs HHT and Con vs both (HHS and HHT). *Rpl11* and *Actb* were found to possess lower stability values than others and therefore, most suitable as normalizer genes according to NormFinder.

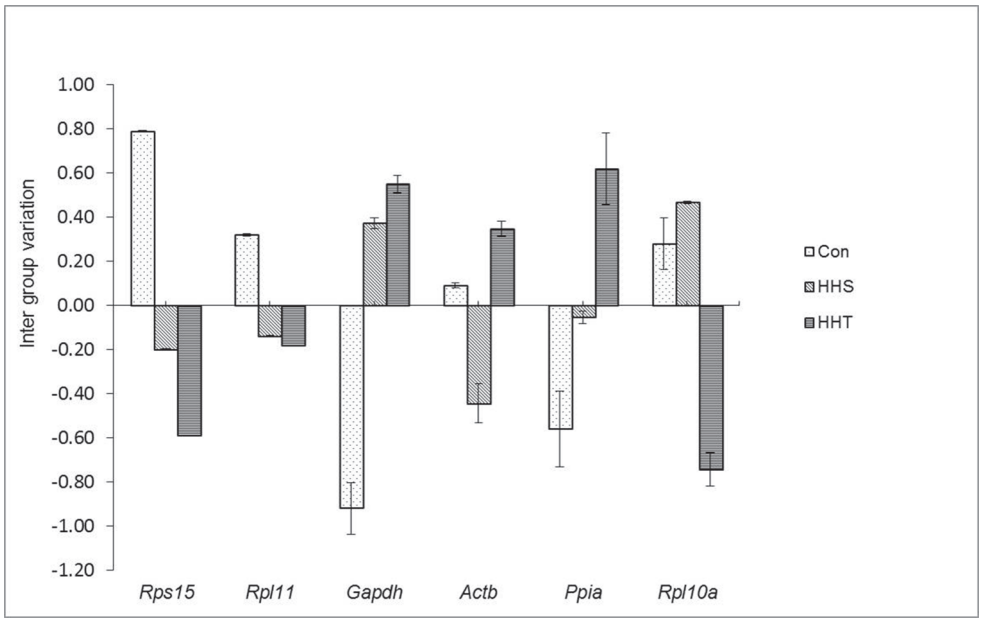

Fig. 3. Bar graph with rectangular bars representing intergroup variation and error bars intra-group variation respectively in combined analysis (Con vs Both (HHS and HHT)), for the stability values obtained using NormFinder. As depicted by NormFinder stability values, *Actb* and *Rpl11* are most suitable reference genes with lower inter-group variability than others. However, *Rps15* and *Rpl11* show lowest intra-group variation.

BestKeeper results suggested that *Rps15, Actb* and *Rpl11* had standard deviation (S.D.) <1 for comparison set Con vs HHS, while S.D.<1 was observed in *Rps15* and *Rpl11* for Con vs HHT and combined (Con vs both (HHS and HHT)) with all results having significant p-value <0.05 and coefficient of correlation close to 1 (Table 2). Therefore, *Rps15* and *Rpl11* were suggested to be the desired reference genes.

In our study, data analysis by GeNorm and BestKeeper showed that *Gapdh* has expression variability and is therefore, unfit for normalization. It was decided to take more than two reference genes namely, *Rpl11*, *Rps15* and *Actb* in compliance with the softwares result, as inclusion of more than one reference gene makes the result statistically more reliable. When the expression of these genes was studied along with other candidates, it was found that *Rpl11* and *Rps15* were showing least variation in their Cq values. Besides that, as explained in below mentioned results, *Rpl11, Actb and Rps15* were found to be suitable normalization factors for acute hypobaric hypoxia irrespective of the susceptible and tolerant rat samples under study.

Differential Expression analysis of HIF-1 and HIF-2.

Geometric mean of Cq values of the genes *Rpl11, Actb and Rps15* was used to calculate the ΔΔCq values for obtaining the fold change in target genes *Hif1*α and *Hif2*α. The average Cq values obtained in each group were first taken as input to obtain average ΔCq values by calculating the mean difference of Cq observed in target and reference genes. Thus, the fold change values obtained after calculating ΔΔCq in each case for *Hif1*α were 0.0208±0.0067 and 0.0189±0.0062 in HHS and HHT rat samples, respectively and similarly, for *Hif2*α the values were 0.833±0.009 in HHS and 0.205±0.017 in HHT rat samples (Figure 4). Thus, level of mRNA expression in both *Hif1*α and *Hif2*α decreased with little or no change in the transcript expression of *Hif2*α in HHS with respect to Con group.

In the present experiment, the protein expression of HIF-1 was observed to be higher in case of *HHS* and *HHT,* when compared to normoxic group. In fact, more intense bands of

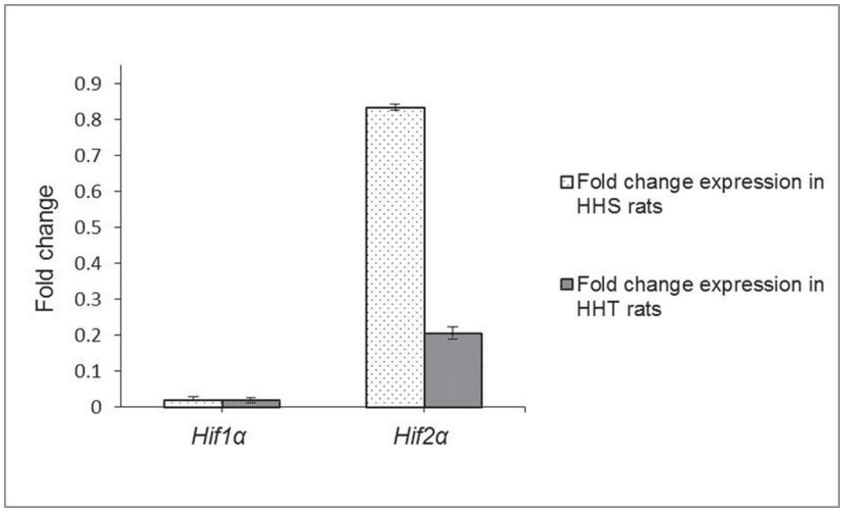

Fig. 4. Graph of relative expression change obtained from expression for *Hif1*α and *Hif2*α in HHS and HHT rats with respect to normoxia control rat sample. Both the genes *Hif1*α and *Hif2*α show decreased expression of mRNA in Hypobaric Hypoxia Susceptible (HHS) as well as Hypobaric Hypoxia Tolerant (HHT) rats when compared to normoxia Control (Con) group. However, *Hif2*α in HHS rats (fold change being 0833±0009) shows only a marginal decrease in the transcript expression levels as compared to Con.

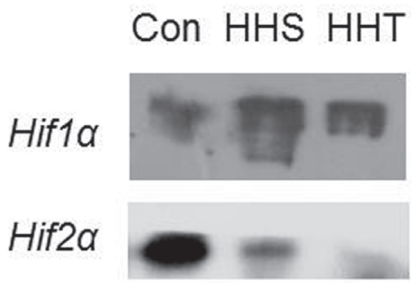

Fig. 5. Blot results showing protein bands of *Hif1*α and *Hif2*α in Con. HHS and HHT samples. Intense bands of *Hif1*α in Hypobaric Hypoxia Susceptible (HHS) as compared to Hypobaric Hypoxia Tolerant (HHT) and Control (Con) rats indicate increased protein expression of *Hif1*α.

HIF-1 represents more accumulation of protein in HHS than HHT rats and probably points towards its substantial requirement to overcome the worsening pathological condition in HHS than HHT rats (Figure 5).

Discussion

Hypobaric hypoxia resulting from oxygen deficiency at higher altitudes may cause various high altitude maladies such as High Altitude Cerebral Edema (HACE) and High Altitude Pulmonary Edema (HAPE) in people who ascend to such regions. If not treated early or the person is not taken immediately to lower altitudes, the

Table 2: BestKeeper software output showing genes selected to be best suitable candidates for normalization with standard deviations <1. Lower standard deviation and higher correlation co-efficient values indicate suitability of genes (*Rpl11, Rps15* and *Actb*).

Con vs HHS	***Gapdh***	***Rps15***	***Actb***	***Ppia***	***Rpl11***	***Rpl10a***
S.D. [± CP]	1.96	0.31	0.63	1.39	0.69	1.16
p-value	0.001	0.001	0.008	0.003	0.001	0.003
Power [x-fold]	3.63	1.22	1.52	2.57	1.58	2.11
Coeff. of correlation [r]	0.989	0.981	0.928	0.953	0.988	0.954
[r^2]	0.978	0.962	0.861	0.908	0.976	0.91
Con vs HHT	***Gapdh***	***Rps15***	***Actb***	***Ppia***	***Rpl11***	***Rpl10a***
S.D.[± CP]	2.57	0.51	1.68	2.36	0.88	1.14
p-value	0.001	0.001	0.001	0.001	0.07	0.001
Power [x-fold]	3.17	1.26	2.15	2.9	1.4	1.67
Coeff. of correlation [r]	0.993	0.996	0.986	0.982	0.776	0.995
[r^2]	0.986	0.992	0.972	0.964	0.602	0.99
Con vs HHS vs HHT	***Gapdh***	***Rps15***	***Actb***	***Ppia***	***Rpl11***	***Rpl10a***
S.D. [± CP]	2.01	0.37	1.24	1.76	0.81	1.05
p-value	0.001	0.001	0.001	0.001	0.001	0.043
Power [x-fold]	3.24	1.25	2.04	2.85	1.65	1.49
Coeff. of correlation [r]	0.989	0.988	0.92	0.97	0.989	0.683
[r^2]	0.978	0.976	0.846	0.941	0.978	0.466

condition can become life threatening. However, certain individuals show variable adaptation response to such diseases as evident from various studies involving human and animal subjects. The susceptibility and tolerance to hypobaric hypoxia in rats has long been studied on the basis of gasping time, measured after exposure of animals to simulated extreme altitude conditions (12). Since, the higher capillary density of rats renders them more tolerant to lower altitudes than humans (22) and in order to maintain the susceptibility and tolerance trait observed at 9754 m in the groups, a simulated altitude of 9144 m was adopted for acute hypobaric hypoxia exposure for a short duration of one hour to avoid unnecessary damage to animals. Since the expression of both *Hif1*α and *Hif2*α genes is associated with adaptation to high altitude, we examined whether *Hif1*α and *Hif2*α are differentially expressed in lungs of hypobaric hypoxia susceptible and tolerant rats during acute and extreme hypobaric hypoxia conditions as compared to normoxia. In order to study the expression of *Hif1*α and *Hif2*α, a set of reference genes *Gapdh*, *Actb*, *Ppia*, *Rpl10a*, *Rpl11* and *Rps15* were evaluated to select best suitable reference genes for normalization in qRT-PCR studies. A few studies on HAPE have used endogenous controls such as β-actin, 18S rRNA (normalization not done) and ASAH1 and MAN1A1 (chosen normalization factors) for qRT-PCR analysis of gene expression of HAPE affected and unaffected individuals (23, 24). However, to the best of our knowledge, there has been no normalization study till date for gene expression studies on lungs of rats exposed to acute hypobaric hypoxia.

Reference Genes Used for qRT-PCR Analysis: *Gapdh* (Glyceraldehyde phosphodehydrogenase) is the most popular gene used as a reference gene in qRT-PCR (25, 26, 27). However, its expression has been shown to vary in different experimental conditions. Since Peptidylprolyl isomerase A or Cyclophilin A (PPIA), is a highly conserved multifunctional enzyme in animals catalyzing cis-trans isomerisation of peptidyl-prolyl bond of proteins and participates in protein folding (28). It seems fit to be considered as reference target and has been found as a suitable reference gene in combination with others in many recently published experiments (27, 20). *Actb* or Beta Actin also has been used as reference gene in many studies such as in up regulation of haemoglobin in alveolar epithelial cell culture exposed to hypoxia (29) and measurement of leptin expression without prior normalization (30). *Rpl10a*, *Rpl11* and *Rps15* genes were chosen because of their essential and conserved roles in ribosome formation. For a functional 80S ribosome, *Rpl10a* is required to assemble 40S and 60S ribosomes and thought to be conserved amongst many eukaryotic species (31). Similarly, *Rpl11* is also required for the production of mature 28S and 5.8S rRNA. In addition, *Rpl11* has been found to be also associated with 5S rRNA during ribosomal biogenesis in yeast (32). Ribosomal protein *Rps15* being highly conserved plays an important role by interacting with 16S rRNA for small subunit central domain assembly (33). Thus, these ribosomal genes seem to be highly promising reference targets and it was expected that they will show more consistent expression in response to acute hypobaric hypoxia.

A number of studies involving cancer cell lines also found altered expression of *Gapdh* (34, 35) and a plausible explanation for its altered and up-regulated expression in cancer cell lines has been provided as presence of hypoxia responsive elements in the upstream region of *Gapdh* (36). The unsuitability of *Ppia* as a reference gene in this experiment might be accounted by the fact that like *Gapdh*, *Hif1*α binding sites or hypoxia responsive elements were also found in its promoter and that, it has been shown to play important role in ROS mediated oxidative stress, inflammatory signalling and vasculo-smooth muscle cell proliferation (37), numerous cancers (38), in COPD pathogenesis (39), and in pathogenesis of various cardiovascular diseases (40). This justifies the significance of our study aimed to select the optimal reference genes in

order to measure gene expression of *Hif1*α and *Hif2*α in pulmonary tissue under the effect of acute hypobaric hypoxia.

In the presence of oxygen and other co-factors, HIF-alpha gets hydroxylated by *Prolyl Hydroxylase Domain-containing* proteins (PHDs) and Factor Inhibiting HIF-1 (FIH-1) at specific proline and asparagine N-803 residues, respectively. This hydroxylated protein subunit binds to pVHL to form E3 ubiquitin ligase VHL complex, which is then rapidly degraded by proteasomal complex. However, during hypoxia, it stabilizes and translocates into the nucleus from cytoplasm, where it binds to ARNT to form HIF complex. HIF then acts as a transcription factor and binds HREs in the regulatory elements of various genes, thereby inducing their gene expression (41).

While a decrease in mRNA expression of *Hif1*α in acute hypoxia has been previously seen and explained possibly due to chromatin modeling in a study involving cell lines exposed to a hypoxia mimetic (42). It was shown using ChIP assay that during hypoxia, the acetylation of histones H3 and H4 present in the proximal region of enhancer/promoter region near the transcription start site of *Hif1*α is decreased, thereby strongly reducing its transcription (42). We found protein expression of HIF-2 markedly decreased in HHT than HHS group when compared to the Con group, which is well correlated with its decreased mRNA expression pattern in hypobaric hypoxia. During its initial discovery, it was found that HIF-2 is markedly expressed in lungs during normoxia and is required for lung and vascular development (43). Mole *et al.* proved that HIF-2 may not contribute to acute hypoxia as it does not show much transcriptional activation of genes containing HREs, despite its high binding affinity for these sites (44). This may partly explain that HIF-1, but not HIF-2 seems to contribute significantly in any response to initial phases of hypobaric hypoxia due to marked decrease in its protein as well as mRNA expression. However, the precise reason of this pattern of expression can be found only by performing some epigenetic studies on HIF-1 and HIF-2. We also suggest that expression of some down targets of these genes and various other factors need to be examined, as these might directly or indirectly play a significant role in tissue protection or deterioration during initial exposure to acute and extreme hypobaric hypoxia.

In conclusion, *Rpl11, Actb* and *Rps15* were found to be the best suitable reference genes to analyse expression of target hypobaric hypoxia responsive genes in our study. Besides this, increased HIF-1 protein expression in HHS and HHT rats, and decreased HIF-2 expression signifies that HIF-1 may play a significant role in the cellular response to hypobaric hypoxia, though, the function of HIF-2 here is still not clear.

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Declaration of Interest

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

References

1. Gallagher, S.A. and Hackett, P.H. (2004) High-altitude illness. Emerg Med Clin N Am. 22: 329-355.
2. West, J.B., Tsukimoto, K., Mathieu-Costello, O. and Prediletto, R. (1991). Stress failure in pulmonary capillaries. J Appl Physiol. 70: 1731-1742.
3. Bartsch, P., Mairbaurl, H., Maggiorini, M. and Swenson, E.R. (2005). Physiological aspects of high-altitude pulmonary edema. J Appl Physiol. 98: 1101–1110.

4. Garbella, E., Catapano, G., Pratali, L. and Pingitore, A. (2011). Pulmonary Edema in Healthy Subjects in Extreme Conditions. Pulm Med. 2011: 1-9.

5. Dehnert, C., Grünig, E., Mereles, D., Von Lennep, N. and Bärtsch, P. (2005). Identification of individuals susceptible to high-altitude pulmonary oedema at low altitude. Eur Respir J. 25: 545-551.

6. Swenson, E.R. and Bärtsch, P. (2012). High-altitude pulmonary edema. Compr Physiol. 2: 2753-2773.

7. Yi X., Liang, Y., Huerta-Sanchez, E., Jin, X., Cuo, ZXP., Pool, J.E. *et al*. (2010). Sequencing of 50 human exomes reveals adaptation to high altitude. Science. 329: 75-78.

8. Julian, C.G., Subudhi, A.W., Hill, R.C., Wilson, M.J., Dimmen, A.C., Hansen, K.C. and Roach, R. C. (2014). Exploratory proteomic analysis of hypobaric hypoxia and acute mountain sickness and in humans. J Appl Physiol. 116: 937-944.

9. Kobayashi, N., Hanaoka, M., Droma, Y., Ito, M., Katsuyam, Y., Kubo, K. and Ota, M. (2013). Polymorphisms of the Tissue Inhibitor of Metalloproteinase 3 Gene Are Associated with Resistance to High-Altitude Pulmonary Edema (HAPE) in a Japanese Population: A Case Control Study Using Polymorphic Microsatellite Markers. PLOS One. 8: 1-7.

10. Keith, B., Johnson, R.S. and Simon, M.C. (2011). HIF1a and HIF2a: sibling rivalry in hypoxic tumor growth and progression. Nat Rev Can. 12: 9-22.

11. Beall, C.M., Cavalleri, G.L., Deng, L., Elston, R.C., Gao, Y., Knight, J., Li, C., *et al*. (2010). Natural selection on EPAS1 (HIF2alpha) associated with low hemoglobin concentration in Tibetan highlanders. Proc Natl Acad Sc U S A. 22: 11459-11464.

12. Shukla, D., Saxena, S., Purushothaman, J., Shrivastava, K., Singh, M., Shukla, S., Malhotra, V. K., Mustoori, S. and Bansal, A. (2011). Hypoxic preconditioning with cobalt ameliorates hypobaric hypoxia induced pulmonary edema in rat. Eur J Pharmacol. 656: 101-109.

13. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009). The MIQE guidelines, minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 55: 611-622.

14. Vandesompele, J., Preter, K.D., Pattyn, F., Poppe, B., Roy, N.V., Paepe, A.D. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:00341–003411.

15. Andersen, C.L., Ledet-Jensen, J. and Ørntoft, T. (2004). Normalization of real-time quantitative RT-PCR data, a model based variance estimation approach to identify genes suited for normalization applied to bladder and colon cancer datasets. Cancer Res. 64: 5245-5250.

16. Pfaffl, M.W., Tichopad, A., Prgomet, C. and Neuvians, T.P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity, BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol Lett. 26: 509–515.

17. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 13:134.

18. Kibbe, W.A. (2007). OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. 35:W43-W46.

19. Livak, K.J. and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$-. Method Methods. 25:402-408.

20. Rienzo, M., Schiano, C., Casamassimi, A., Grimaldi, V., Infante, T. and Napoli, C. (2013). Identification of valid reference housekeeping genes for gene expression analysis in tumor neovascularization studies. Clin Transtl Oncol. 15:211-218.

21. Wang, M., Wang, Q. and Zhang, B. (2013). Evaluation and selection of reliable reference genes for gene expression under abiotic stress in cotton (*Gossypium hirsutum* L). Gene. 530: 44-50.

22. Shrivastava, K., Shukla, D., Bansal, A., Sriram, M., Banerjee, P.K. and Ilavazhagan, G. (2008). Neuroprotective effect of cobalt chloride on hypobaric hypoxia-induced oxidative stress. Neurochem Int. 52: 368–375.

23. Mairbäurl, H., Schwöbel, F., Höschele, S., Maggiorini, M., Gibbs, S., Swenson, E. R. and Bärtsch P. (2003). Altered ion transporter expression in bronchial epithelium in mountaineers with high-altitude pulmonary edema. J Appl Physiol. 95: 843-1850.

24. Ali, Z., Mishra, A., Kumar, R., Alam, P., Pandey, P., Ram, R. *et al*. (2012). Interactions among Vascular-Tone Modulators Contribute to High Altitude Pulmonary Edema and Augmented Vasoreactivity in Highlanders. PLoS ONE. 7: e44049.

25. Wisnieski, F., Calcagno, D.Q., Leal, M.F., dos Santos, L.C., de Oliveira Gigek, C., Chen, E.S. *et al* (2013). Reference genes for quantitative RT-PCR data in gastric tissues and cell lines World. J Gastroentero. 19: 7121.

26. Zhang, L., He, L.L., Fu, Q.T. and Xu, Z.F. (2013). Selection of Reliable Reference Genes for Gene Expression Studies in the Biofuel Plant Jatropha curcas Using Real-Time Quantitative PCR. Int J Mol Sci. 14: 24338-24354.

27. Sirakov, M., Borra, M., Cambuli, F.M. and Plateroti, M. (2013). Defining Suitable Reference Genes for RT-qPCR Analysis on Intestinal Epithelial Cells. Mol Biotechnol. 54: 930-938.

28. Frausto, S.D., Lee, E. and Tang, H. (2013). Cyclophilins as modulators of viral replication. Viruses. 5: 1684-1701.

29. Grek, C.L., Newton, D.A., Spyropoulos, D.D. and Baatz, J.E. (2011). Hypoxia Up-Regulates Expression of Hemoglobin in Alveolar Epithelial Cells. Am J Resp Cell Mol Biol. 44: 439-447.

30. Meißner, U., Hanisch, C., Ostreicher, I., Knerr, I., Hofbauer, K. H., Blum, W. F., Allabauer, I., Rascher, W. and Dotsch, J. (2005). Differential Regulation of Leptin Synthesis in Rats during Short-Term Hypoxia and Short-Term Carbon Monoxide Inhalation. Endocrinol. 146: 215–220.

31. Del Campo, E.M., Casano, L.M. and Barreno, E. (2013). Evolutionary implications of intron-exon distribution and the properties and sequences of the RPL10A gene in eukaryotes. Mol Phylogenet Evol. 66:857-867.

32. Zhang, J., Harnpicharnchai, P., Jakovljevic, J., Tang, L., Guo, Y., Oeffinger, M., Rout, M.P., Hiley, S.L., Hughes, T. and Woolford, Jr. J.L. (2007). Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins Rpl5 and Rpl11 into nascent ribosomes. Gene Dev. 21: 2580–2592.

33. Serganov, A., Polonskaia, A., Ehresmann, B., Ehresmann, C. and Patel, D.J. (2003). Ribosomal protein S15 represses its own translation via adaptation of an rRNA-like

fold within its mRNA. EMBO J. 22:1898-1908.

34. Silverthorn, C.F. and Alani, R.M. (2010). Glyceraldehyde-3-phosphate dehydrogenase expression is altered by hypoxia in melanoma cells and primary human melanocytes. Melanoma Res. 20: 61-63.

35. Higashimura, Y., Nakajima, Y., Yamaji, R., Harada, N., Shibasaki, F., Nakano, Y. and Inui H. (2011). Up-regulation of glyceraldehyde-3-phosphate dehydrogenase gene expression by HIF-1 activity depending on Sp1 in hypoxic breast cancer cells. Arch Biochem Biophys. 2011; 509:1-8.

36. Lu, S., Gu, X., Hoestje, S. and Epner, D.E. (2002). Identification of an additional hypoxia responsive element in the glyceraldehyde-3-phosphate dehydrogenase gene promoter. Biochimic Biophysic Acta. 1574: 152-156.

37. Satoh, K., Nigro, P. and Berk, B.C. (2010). Oxidative Stress and Vascular Smooth Muscle Cell Growth, A Mechanistic Linkage by Cyclophilin A. Antioxid Red Sign. 12: 675–682.

38. Hu, R., Ouyang, Q., Dai, A., Tan, S., Xiao, Z. and Tang, C. (2011). Heat shock protein 27 and cyclophilin A associate with the pathogenesis of COPD. Respirology. 16: 983-993.

39. Lee, J. and Kim, S.S. (2010). Current implications of cyclophilins in human cancers. J Exp Clin Cancer Res. 29: 1-6.

40. Satoh, K., Shimokowa, H. and Berk, B.C. (2011) Cyclophilin A Promising New Target in Cardiovascular Therapy. Circulation J. 74:2249-2256.

41. Ke, Q. and Costa, M. (2006). Hypoxia-inducible factor-1. Mol Pharmacol. 2006; 70: 1469-1480.

42. Lin, Q., Cong, X. and Yun, Z. (2011). Differential hypoxic regulation of hypoxia-inducible factors 1a and 2a. Mol Cancer Res. 9:757-765.

43. Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y. and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1a regulates the VEGF expression and is potentially involved in lung and vascular development. Proc Natl Acad Sci USA. 1997; 94: 4273-4278.

44. Mole, D. R., Blancher, C., Copley, R. R., Pollard, P.J., Gleadle, J.M., Ragoussis, J. and Ratcliffe, P. J. (2009). Genome-wide association of hypoxia-inducible factor (HIF)-1a and HIF-2a DNA binding with expression profiling of hypoxia-inducible transcripts. J Biol Chem. 284: 16767-16775.

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Identification of Soil Enriched Microorganisms using 16S rDNA analysis for Crop Prodctivity

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Abstract

In the present study, occurrence of cultivable bacteria in the soils of Chandan plant (*Santalum album*) and Bamboo plant (*Bambussa bambus*) was investigated. Soils are likely to include species of various bacterial phyla, so different colony characteristics isolates were selected and 16S rDNA sequences were amplified from the samples. Five genera (MS2) and 3 genera (MS1) were identified by 16S ribosomal RNA gene sequence analysis (similarities to known 16S rDNA sequences). The majority of bacteria were *Bacillus* belonged to Firmicutes in MS2. While the presence of *Pseudomonas, Micrococcus* and Enterobacter were less. The endophytic bacterial community was also present in MS1 sample. In addition, phylogenetic analyses were also used to infer the makeup of bacterial communities in the soil samples. As a result, our isolates (from both soil) nicely clustered with reference sequences (16S rDNA sequences). Our results show that the population diversity of cultivable bacteria is abundant in the both soil types. These cultivable bacterial consortium may be used in crop production.

Key words: Bamboo; Chandan 16 s RNA gene; Soil

Introduction

The increasing need for environmental friendly agricultural practices is driving the use of fertilizers based on beneficial microorganisms. Microorganism belongs to a wide array of genera, classes, and phyla ranging from bacteria to yeasts and fungi, which can support plant nutrition with different mechanisms (1). Moreover, studies on the interactions between plant and soil with different microorganisms are shedding light on their interrelationships thus providing new possible ways to exploit them for agricultural purposes. Organic fertilizers have been introduced in the recent years, which are also acting as natural stimulators of plant growth and development (2, 3). A specific group of this kind of fertilizers includes products containing living cells of different types of plant growth-promoting microorganisms (PGPM) which when, applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting nutritionally important elements (nitrogen, phosphorus, etc.,) from unavailable to available form through biological process such as nitrogen fixation and solubilization of rock phosphate (4). Beneficial microorganisms in biofertilizers accelerate and improve plant growth and protect plants from pests and diseases (5). These potential microorganism act as a biofertilizers and play a key role in productivity and sustainability of soil and also protect the environment as eco-friendly and cost effective inputs for the farmers. With using the biological and organic fertilizers, a low input system can be carried out and it can be help achieving sustainability of farms (6).

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (7). Among the bacterial genera with this capacity are *Pseudomonas, Bacillus, Micrococcus.* The present study was aimed to examine the diversity of bacteria in two soil of Modasa, Gujarat India. The molecular techniques now available were used to sequence 16S rDNA gene of bacteria, and a phylogenetic tree for 16S rDNA sequences.

Materials and Methods

***Collection of Experimental Material*:** The soil used in this study was collected from agricultural land of Modasa (MS1 and MS2), Gujarat as per the method of (8). Two different types of soil samples was collected in sterile container and immediately transported at 4^{0}C in cool pack to the microbiology laboratory Xcelris Genomics, Ahmedabad, India. The soil samples were collected at around 9.00 am from four different places of Modasa (23.47°N 73.3°E), Gujarat, India (Fig. 1).

***Sample Preparation*:** Ten gm of each soil samples were dissolved in 100ml of PBS buffer and kept on rotatary shaker overnight at 37^0 C. Further, each sample was diluted in sterile water to $10^{-6.}$ From each dilution, a 100 µl aliquots was plated out on selective medium.

Media preparation: The media used to grow culturable bacteria is Luria Bertoni agar or Broth Medium. Ready to use media were purchased from Hi-media used for isolation of Rhizosphere bacteria.

***Culture Conditions*:** Each serially diluted sample were plated on Luria Bertoni agar, incubated at 37°C for 24 hrs. After incubation pure colonies streaked out and sub-cultured on

Fig. 1. Location of soil sampling site at Modasa, Gujarat, India.

Nutrient Agar. The presence/absence of different bacterial colonies were evaluated. The presence/absence of microorganisms were determined using the dilution method (9). Dilution of 10^{-6} were prepared and plated on the nutrient medium (10).

Gram Staining: Grams staining of isolated microbial cultures were carried out using crystal violet as described by Jaksi *et al.* (11), Mandri and Lin (12) and Anon (13) with some modifications followed by counter staining with Gram's safranin solution for 60 seconds. Excess safranin solution from slide was washed with water and air-dried. Slide was examined under a microscope (oil immersion objective) and morphology was recorded.

DNA Isolation and PCR Amplification**:** DNA isolation from pure isolate was done using XcelGen Bacterial gDNA kit (Cat # XG2411-01) as per protocol recommended by manufacturer, its quality was evaluated on 1.2% Agarose gel (14). The 16S rDNA gene was amplified using universal primer 8F (5'-AGAGTTTGATC CTGGCTCAG-3'), 1492R (5' TACGGYTACCT TGTTACGACTT-3'). PCR was carried out in a final reaction volume of 25 µl in 200 µl PCR tube Xcelgen Premix 2.0 (Xcelris Labs Ltd., Ahmedabad) containing Taq DNA polymerase (0.625 units), DNTPs (5µM), $MgCl_2$ (0.0375mM) and reaction buffer along with150 ng of template DNA and 10 pmol forward and reverse primer. Amplification was carried out in thermal cycler (eppendorf, Hamburg, Germany) programmed at 1 Cycle of 3 mins at 95°C, 30 cyclers of 30 sec at 95°C, 52°C -16S primers for 30 sec and 72°C for 60 sec, and a final extension step of 72°C for 7 mins. 16S amplicon product were resolved on 1.2 % agarose gel. The image was captured using BIO-RAD gel documentation system.

Sequencing: 16S PCR product (about 1500 bp) of the isolate was sequenced in both directions at Xcelris Labs Ahmedabad. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser.

Sequence analyses and phylogenetic tree constructing: The 16S rDNA gene sequence was analysed using BLAST with nr database of NCBI GenBank database. All reference sequences were obtained from the GenBank/ EMBL/DDBJ/RDP (Benson et al. 2007). The sequences were compared with those in the GenBank databases (www.ncbi.nlm.nih.gov/ blast) by using the BLAST (15). Sequence similarities were determined by using the software package ClustalX (16). Phylogenetic tree was constructed by the neighbour joining method using MEGA 5.0 software (17). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies (18).

Nucleotide sequence accession numbers**:** The partial 16S rRNA gene sequences obtained in this study have been deposited in GeneBank databases under the accession numbers KC309688, KC315760 to KC315761, KC335294 to KC335302 and KC342873 to KC342876, JX843779.

Results

Isolation and identification of bacteria and sequence similarity**:** Sixteen bacterial strains with different colony characteristics were isolated on LB media:11 isolates from the MS2 (bamboo plant) and 6 (Chandan plant) from the MS1 (Tables 1 and 2). All 16 bacterial isolates were sequenced and subjected to sequence analysis, followed by homology search using databases: the GenBank and the Ribosomal Database Project (RDP) database. In our library, all isolate had 100% similarity to 16S rDNA data sequences from those databases (Table1 and 2). All of the bacterial isolates were assigned to three phyla within the domain Bacteria, namely Proteobacteria, Firmicutes, and Actinobacteria (Tables 1 and 2 and Fig. 2 and 3).

Phylogenetic analysis**:** The bacterial isolates of MS1 were represented by only one phyla and

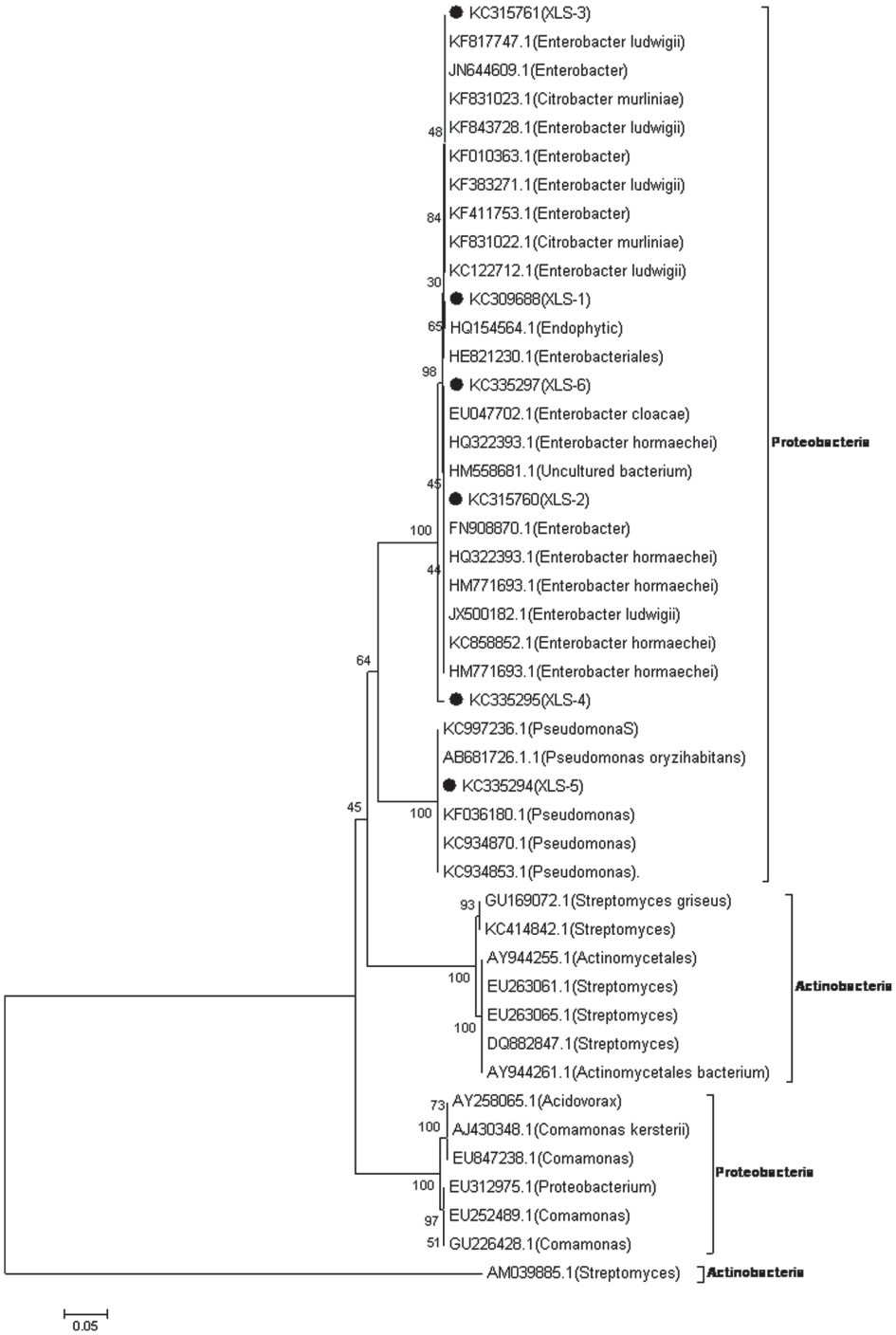

Fig. 2. Phylogenetic relationships of partial 16S rDNA sequences of clones recovered from MS1 1 sample. The tree was inferred by the neighbour joining method using the MEGA 5 software. The scale bar equals to an average of 5 nucleotide substitutions per 100 positions.

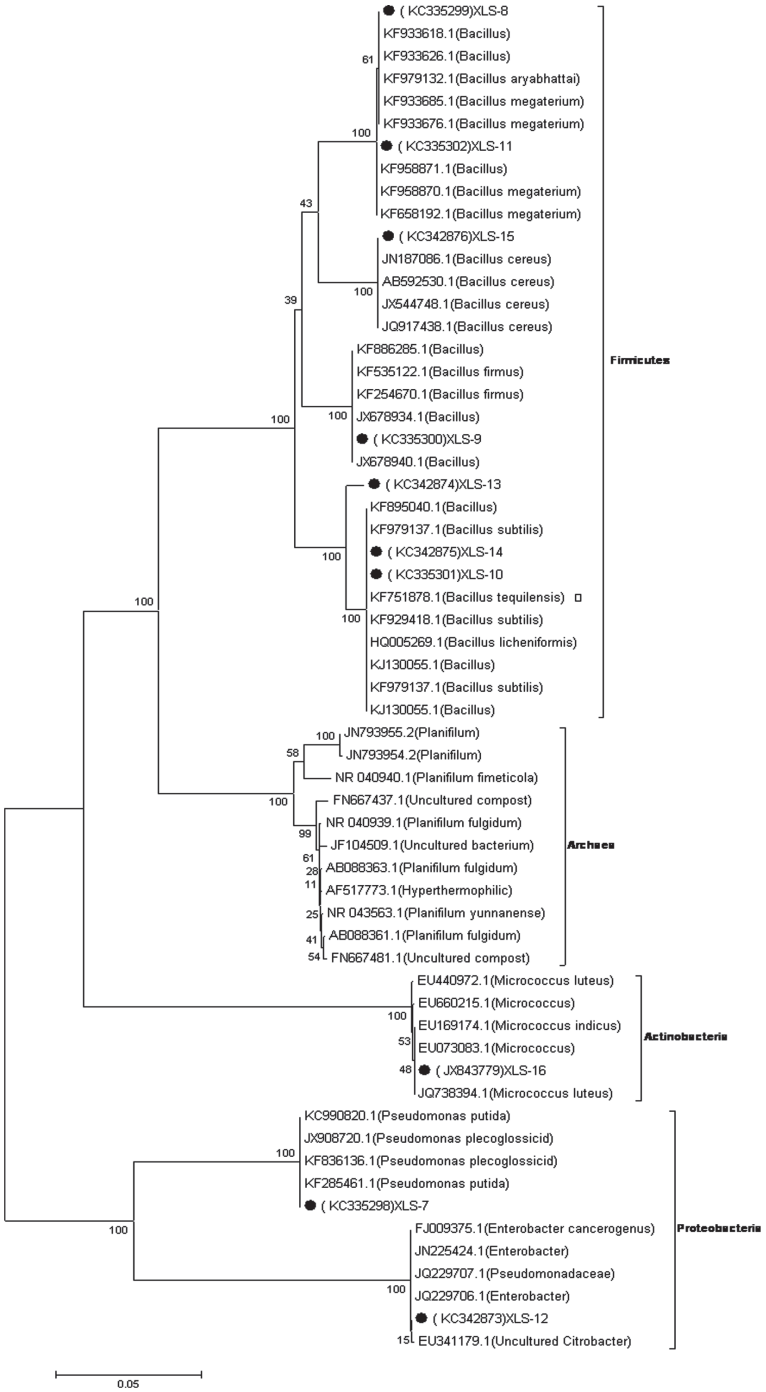

Fig. 3. Phylogenetic relationships of partial 16S rDNA sequences of clones recovered from MS2 sample. The tree was inferred by the neighbour joining method using the MEGA 5 software. The scale bar equals to an average of 5 nucleotide substitutions per 100 positions.

all six isolates falling within the Proteobacteria groups. All the bacterial isolates from rhizosphere were assigned to 3 genera. The three bacterial genera namely Enterobacter (predominant) Pseudomonas and Endophytic were identified in present sample representing 100% of the isolates from the Chadian plants as shown in Table 1 and Fig. 2. The phyla: Firmicutes, Proteobacteria and Actinobacteria were predominant in soil sample 2 (Bamboo soil) and assigned to three genera, among them the members of the genus Bacillus share major taxon (Table 2 and Fig 3).

Discussion

Living organisms both plants and animals, constitute an important component of soil. The pioneering investigations of a number of early microbiologists showed for the first time that the soil was not an inert static material but a medium pulsating with life. Microbes in the soil are important to us in maintaining soil fertility / productivity, cycling of nutrient elements in the biosphere and sources of industrial products such as enzymes, antibiotics, vitamins, hormones, organic acids etc. At the same time certain soil microbes are the causal agents of human and plant diseases.

In the present study, we examined cultured bacterial composition by PCR-based analysis of bacterial 16S rDNA sequences. This culture-dependent method offers possibility of

Table1. Similarity Value of isolates based on 16S r DNA sequences retrieved from the MS1 sample.

Name of Isolates with Accession no.	Sequence Identity(%)	Nearest type Strain with Accession no.	Phylum
XLS-1(KC309688)	100%	Endophytic bacterium(HQ154564)	Proteobacteria
XLS-2(KC315760)	100%	Enterobacter hormaechei(HQ322393)	
XLS-3(KC315761)	100%	Enterobacter ludwigii(KF817747)	
XLS-4(KC335295)	100%	Enterobacter(FN908870)	
XLS-5(KC335294)	100%	Pseudomonas oryzihabitans(AB681726.1)	
XLS-6(KC335297)	100%	Enterobacter cloacae(EU047702.1)	

Table 2. Similarity Value of isolates based on 16S r DNA sequences retrieved from the MS2 sample.

Name of Isolates with Accession no	Sequence Identity(%)	Nearest type Strain with Accession no.	Phylum
XLS-7(KC335298)	100	*Pseudomonas putida* (KC990820)	Proteobacteria
XLS-8(KC335299)	100	*Bacillus sp.* (KF933626)	Firmicutes
XLS-9(KC335300)	100	*Bacillus firmus* (KF535122)	
XLS-10(KC335301)	100	*Bacillus tequilensis* (KF751878.1)	
XLS-11(KC335302)	100	*Bacillus megaterium* (KF958870)	
XLS-12(KC342873)	99	*Enterobacter sp.*(JQ229706)	Proteobacteria
XLS-13(KC342874)	100	*Bacillus licheniformis* (HQ005269)	Firmicutes
XLS-14(KC342875)	100	*Bacillus subtilis* (KF979137)	
XLS-15(KC342876)	99	*Bacillus cereus* (JN18708)	
XLS-16(JX843779)	100	*Micrococcus luteus* (JQ738394)	Actinobacteria

characterizing microbial ecosystems, independent isolation, maintenance, and propagation of bacteria under laboratory conditions. However, PCR-based methodologies are subject to certain limitations (19). As discussed by Wintzingerode et al. (20), care should be taken in experimental procedures and in interpretation of the results. Thus our findings reveal that both soil contained a diverse array of bacterial species. The cultivable bacterial isolates obtained in this study represent known bacterial genera from the phyla Proteobacteria, Firmicutes and Actinobacteria.

The soil contains various compounds, like amino acids, proteins, carbohydrates, vitamins and hormones, which are important sources for the microorganisms' diversity (21, 22). We found several culturable bacteria in the rhizosphere of bamboo and chandan plant. Similar results have been reported previously for sugarcane (23), maize, and coffee plants (24).

The culturable bacterial isolates obtained in this study represent a few known bacterial genera. *Bacillus* and *Pseudomonas* species were the dominant isolated bacteria in the rhizosphere of both soil types. Similar results have also been reported by Han et al. (22) in bamboo soil in China. Tamilarasi et al. (25) isolated various bacteria such as *Bacillus, Pseudomonas, Enterobacter, Corynebacterium, Micrococcus* and *Serratia* from rhizosphere of medicinal plants. The main reason of microbial specificity towards the plants could be due to exchange of plant metabolites (26). Production of phytohormones by these microbial species may affect plant growth and development (27,28).

Fertile soil contains huge amount of phosphorus that exists in insoluble forms. Bacteria Enterobacter sp. may have the capability to solubilize insoluble phosphorus and hydrolyze for plant growth (29). Enterobacter species was also observe in the present study which could act as phosphate-solubilizing bacteria (PSB) and help in crop production. Antimicrobial producing properties of Enterobacter have been reported by Mandal et al. (30) from soil sample.

Pseudomonas are considered as important constituents in the root-associated microbial community, and their ability to colonize the root surface, preventing the development of plant pathogens and improving plant growth, is well known (31, 32, 33). Endophytic bacteria was also present in present study. The dominating roles of plants in the control of the endophytic bacterial community composition have also been observed (34, 35). On the other hand, it is generally assumed that many bacterial endophyte communities are the products of a colonizing process initiated in the root zone (21).

This study provides basic information about the diversity of bacteria associated with bamboo and chandan plant. Results obtained in this work to help in design a microbial consortium for plant growth development. Further investigation, including efficiency test under field condition with various plants is needed to clarify the role of PGPR and their beneficial effects on plant growth and development.

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References

1. Malusa, E., Sas-Paszt, L. and Ciesielska J. (2012). Technologies for Beneficial Microorganisms Inocula Used as Biofertilizers. World. Scientific. J., 2012: 12.
2. Khan, W., Rayirath, U.P. and Subramanian, S. (2009). Seaweed extracts as bio stimulants of plant growth and development. J. Plant. Growth. Regul., 28(4): 386–399.
3. Gousterova, A., Nustorova, M. and Christov, P. (2008). Development of a biotechnological procedure for treatment of animal wastes to obtain inexpensive biofertilizer. World. J. Microb. Biot., 24(11): 2647–2652.

4. Rokhzadi, A., Asgharzadeh, A. and Darvish, F. (2008). Influence of plant growth-promoting rhizobacteria on dry matter accumulation and yield of chickpea (*Cicer arietinum* L.) under field condition. Am-Euras. J. Agric. Environ. Sci. 3(2): 253-257.

5. El-Yazeid, A.A., Abou-Aly, H.A. And Mady, M.A. (2007). Enhancing growth, productivity and quality of squash plants using phosphate dissolving microorganisms (bio phosphor) combined with boron foliar spray. Res. J. Agric. Biol. Sci., 3(4): 274-286.

6. Mohammadi, K. and Sohrabi, Y. (2005). Bacterial Biofertilizer For Sustainable Crop Production. A review. ARPN. J. of Agricult. and Biolog. Science. 7(5): 307-316.

7. Goldstein, A.H. (1986). Bacterial solubilization of mineral phosphates: historical perspective and future prospects. Am. J. Altern. Agri., 1: 51–7.

8. Dutta, M., Sardar, D., Pal, R. and Kole, R.K. (2010). Effect of Chlorpyrifos on microbial biomass and activities in tropical clay loam soil. J.Environ Monit Assess., 160, 385-391.

9. Ajaz, M., Jabeen, N., Akhtar, S., and Rasool, S.A. (2005). Chlorpyrifos resistant bacteria from Pakistani soils: Isolation, Identification, resistance profile and Growth Kinetics. Pakistan. J Bot., 37(2): 381-388.

10. Onet, A. (2008). Research concerning changes of the microbiological characteristics and biological activity of preluvosoil under different treatment with pesticides and chemical fertilizer management; International Symposia Risk Factor for Environment and Food Safety and Natural Resources and Sustainable Development, Faculty of Environmental Protection, Oradea.

11. Jaksic, S., Uhitil, S. and Zivkovic (1998). Arhi. grad. Toksilkol., 49: 239-255.

12. Mandri, T. and Lin, J. (2006). Afric. J. of Biotechnol. 6 (1): 23-27.

13. Anon. (1957). Manual of microbiological methods. Mc-Graw Hill Book Co., New York, pp. 127.

14. Peixoto, R. S., Coutinho, H. L. C. and Rumjanek, N. G. (2002). Use of rpoB and 16S rDNA genes to analyse bacterial diversity of a tropical soil using PCR and DGGE. Letters. in Appl. Microbiol. 35: 316-320.

15. Madden, T.L., Tatusov and R.L., Zhan, J. (1996). Application of network BLAST server. Methods. in Enzymol., 266: 131–141.

16. Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids. Res. 22: 4673–4680.

17. Tamura, K., Dudley, J. and Nei, M. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA 4.0). J. Mol. Bio. Evol. 24:1596-1599.

18. Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39: 783-791.

19. Acinas, S.G., Sarma, R. and Klepac. V. (2005). PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Appl. Environ. Microbiol., 71: 8966–8969.

20. Wintzingerode, V.F., Goebel, U.B. and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS. Microbiol. Rev. 21: 213–229.

21. Compant S., Duffy, B., Nowak, J., Clement, C. and Barka, E.A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of

action, and future prospects. Appl. Environ. Microbiol., 71: 4951–4959.

22. Han, J., Sun, L. and Dong, X. (2005). Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. Syst. Appl. Microbiol., 28: 66–76.

23. Mendes, R., Pizzirani-Kleiner, A.A. and Araujo, W.L. (2007). Diversity of cultivated endophytic bacteria from sugarcane: genetic and biochemical characterization of Burkholderia cepacia complex isolates. Appl. Environ. Microbiol. 73:7259–7267.

24. Estrada-De, L.S.P., Bustillos-Cristales, R. and Caballero-Mellado, J. (2001). Burkholderia, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl. Environ. Microbiol., 67: 2790–2798.

25. Tamilarasi, S., Nanthakumar, K. and Karthikeyan, K., (2008). Diversity of root associated microorganisms of selected medicinal plants and influence of rhizo microorganisms on the antimicrobial property of *Coriandrum sativum*. J Environ Biol.29,127-134.

26. Ramesh, G., VedhaHari, B.N. and Dhevendaran, K. (2012). Microbial association with selected medicinal plants in rhizosphere and their biodiversity. Adv. Natur. Appl. Sci., 6: 947-958.

27. Lucas-Garcia, J.A., Probanza, A., Ramos, B. (2004). Effect of inoculation of Bacillus licheniformis on tomato and pepper. Agronomie, 24:169–176.

28. Mena-Violante, H.G., Olalde-Portugal, V. (2007). Alteration of tomato fruit quality by root inoculation with plant growth-promoting rhizobacteria (PGPR) Bacillus subtilis BEB-13bs. Sci. Hort. 113: 103–106.

29. Kil Yong, K., Jordan, D., McDonald, G.A. (1998). *Enterobacter agglomerans*, phosphate solubilizing bacteria, and microbial activity in soil: Effect of carbon sources. Soil. Biolo. and Biochem., 30(8–9): 995–1003.

30. Mandal, S. M., Sharma, S., and Pinnaka, A. K. (2013).Isolation and characterization of diverse antimicrobial lipopeptides produced by Citrobacter and Enterobacter. BMC Microbiol. 13:152.

31. Fett, W.F. (2006). Inhibition of *Salmonella enterica* by plant associated pseudomonads *in vitro* and on sprouting alfalfa seed. J. Food. Prot., 69: 719–728.

32. Park, M.S., Jung, S.R. and Lee, M.S. (2005). Isolation and characterization of bacteria associated with two sand dune plant species, *Calystegia soldanella* and *Elymus mollis*. J. Microbiol. 43: 219–227.

33. Rangarajan, S., Loganathan, P. and Saleena, L.M. (2001). Diversity of pseudomonads isolated from three different plant rhizospheres. J. Appl. Microbiol. 91:742–749.

34. Germida, J.J., Siciliano, S.D. and Freitas, J. R. (1998). Diversity of root-associated bacteria associated with field-grown canola (Brassica napus L.) and wheat (Triticum aestivum L.). FEMS. Microbiol. Ecol., 26: 43–50.

35. Siciliano, S.D., Germida, J.J. (1999). Taxonomic diversity of bacteria associated with the roots of field-grown transgenic Brassica napus cv. Quest, compared to the non-transgenic B. napus cv. Excel and B. rapa cv. Parkland. FEMS. Microbiol. Ecology. 29: 263–272.

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Microemulsion based Hydrogel of Mycophenolate Mofetil for the Treatment of Psoriasis

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Abstract

Mycophenolate mofetil (MMF), the morpholinoethyl ester of mycophenolic acid is an immunosuppressive agent used to prevent organ rejection after kidney and heart transplant. The drug seem to be effective in dermal diseases mainly psoriasis. However, till date it can be only be administered using systemic route which is often associated with side effects such as nausea, leucopenia, sepsis and diarrheoa. The aim of the present study was to develop microemulsion based hydrogel for topical delivery of mycophenolate mofetil and to investigate its *in vitro* release and its potential in treating psoriatic inflammation using imiquimod induced skin inflammation animal model. Pseudoternary phase diagrams were constructed and on the basis of microemulsion existence range, various formulations were developed using oleic acid, Tween80, propylene glycol and distilled water as oil phase, surfactant, cosurfactant and aqueous phase, respectively. The selected formulations were subjected to physical stability studies and consequently to various physicochemical characterization. The optimized formulation (F2) consisting of 6.06% *v/v* of oleic acid 36.36% *v/v* of Tween80 and 18.18% *v/v* of propylene glycol has shown a globule size of 124 nm, refractive index 1.421, zeta potential -34.35 ±0.051 mV, pH 5.9 and conductivity value of 10^4 μS cm^{-1}. The permeability of drug from microemulsion after 24 h was observed to be 69.52%. Carbopol 940 was used to convert microemulsion into microemulsion based hydrogel to improve its viscosity for topical administration and was characterized. The histopathological studies performed on mice skin revealed that the treated skin showed complete clearance of hyperkeratotic plaques and a significant reduction in the area of perakeratosis. The results indicated that the formulated gel may be a promising vehicle for topical delivery of mycophenolate mofetil for the treatment of psoriasis.

Keywords: Microemulsion, mycophenolate mofetil, psoriasis, topical drug delivery

Introduction

Psoriasis is a chronic inflammatory skin disease of unknown etiology characterized by epidermal hyperproliferation, inflammation and altered keratinization (1). Different treatments are available for psoriasis and among this topical therapy are most commonly used in majority of patients. However, efficacy of topical therapy has been a major concern due to skin changes that occur in psoriasis such as imbalanced skin lipids, excessive growth and aberrant differentiation and skin sensitivity. Apart from this, skin rigidization occurs as normal moisturizing factors like water are absent in psoriatic skin, posing a stiff challenge in designing a novel topical delivery system (2).

The barrier nature of skin made it difficult for most of drugs to penetrate into or through it. Many strategies have been employed to enhance dermal and transdermal drug delivery. The common method employed to improve drug permeation involves the use of permeation

enhancers such as organic solvents like ethanol or fatty acids. However, these compounds often lead to generation of skin irritation (3). Other method involves the use of ultrasound and iontophoresis but these methods are not commonly used due to requirement of qualified staff for its application (4). Recently, attempts have been made for the topical delivery of various drugs employing novel colloidal carriers such as microemulsions, nanoemulsions and liposomes.

Microemulsions can be considered as ideal liquid vehicles for drug delivery as they possess most of requirements for this including thermodynamic stability, ease of formulation, low viscosity and high solubilization capacity. Due to small droplet size and large amount of inner phase in microemulsions, the surface area and density of microemulsion droplets are assumed to be very high, providing high concentration gradient and improved drug permeation (5). Microemulsions have been reported to enhance the drug permeation through skin as compared to conventional formulations such as gels and creams (6). Studies have revealed the potential of microemulsion in increasing the skin permeation of various drugs such as triptolide, estradiol, and 8- methoxaslen (7, 8, 9). Microemulsions present advantages over liposomal carriers such as higher stability, low preparation cost, absence of organic solvents and no necessity of intensive sonication (4). The viscosity of microemulsions can also be increased by addition of hydrogels which can prolong their skin retention making them suitable for topical drug delivery (10).

Mycophenolate mofetil (MMF), a prodrug of mycophenolic acid, is a new immunosuppressive compound mainly used in combination of cyclosporine and corticosteroids for the prevention of organ rejection after allogenic heart and kidney transplantations. This substance reversibly blocks the *de novo* synthesis of guanine nucleotides required for the DNA and RNA synthesis in T and B lymphocytes (11). Recently, reports have been published concerning the use of mycophenolate mofetil for the treatment of several autoimmune and inflammatory skin disorders including psoriasis (12). However the systemic administration in dermal therapy is limited due to several side effects such as nausea, leucopenia, sepsis and diarrhea. So, studies are being conducted for the development of suitable topical formulation of MMF. Studies have revealed that MMF is capable of penetrating into skin (13). Furthermore, the biotransformation of the product into its active form in the skin has also been reported (14). Case studies concerning topical application of MMF in patients with plaque type psoriasis showed same effects as topical corticosteroids in controlling erythema and inflammation indicating that MMF could be a promising alternative in the local treatment of psoriasis. The aim of the present study was to develop and evaluate microemulsion based gel system of MMF for the treatment of psoriasis, which could provide improved drug permeation through skin and improved patient compliance.

Materials and Methods

***Material*:** Mycophenolate mofetil was kindly gifted by Panacea Biotec Ltd. Baddi, India. Oleic acid was purchased from Loba Chemie Pvt. Ltd, Mumbai. Soyabean oil, isopropyl palmitate, isopropyl myristate, Tween80, Tween20 and propylene glycol were purchased from SD Fine Chemicals Ltd, Mumbai. Carbopol 940 was obtained from Qualikems Fine Chemicals Pvt. Ltd., Vadodara. Imiquimod cream (Glenmark Pvt. Ltd., Mumbai) was purchased from market. Distilled water (Rions, India) was used throughout the studies. All other chemicals used were of analytical grade.

Screening of Excipients: The solubility of drug in various oils (isopropyl palmitate, isopropyl myristate, oleic acid and ethyl oleate), surfactants (Tween80 and Tween20) and cosurfactant (propylene glycol) was determined by dissolving excess amount of MMF in 5 ml of each selected oils, surfactants and cosurfactants in stoppered vials separately and mixed using vortex mixer (IKA, Germany). Mixtures were then shaken for 72 h in an isothermal shaker (Narang Scientific

Works Pvt. Ltd., India) maintained at 37±1ºC to achieve equilibrium (15). The equilibrated samples were then removed from the shaker, centrifuged at 5000 rpm for 15 minutes to remove the excess amount of undissolved drug. The supernatant was filtered through a 0.45 µm membrane filter and concentration of MMF was determined in each of the selected oil, surfactant and cosurfactant by Ultraviolet Spectrophotometer (Blue Star AU-2701, India) at λ_{max} of 250nm after appropriate dilutions with methanol (16).

Construction of Pseudo-Ternary Phase Diagrams: Pseudo-ternary phase diagrams were constructed so as to find out the concentration range of components for the existence range of microemulsions, using water titration method (17) at ambient temperature (25ºC). Three phase diagrams were prepared with 1:1, 2:1 and 3:1 volume ratios of Tween80 and propylene glycol, respectively. For each phase diagram, oil and specific surfactant/ cosurfactant (S_{mix}) ratio were mixed thoroughly in different combinations of oil and S_{mix} (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1) so that the maximum ratios could be covered for the study to delineate the boundaries of phases formed precisely in the phase diagrams. Slow titration with distilled water was carried out for each specific ratio of oil and S_{mix} under moderate stirring. After equilibration, the mixtures were assessed visually and determined as being microemulsions, crude emulsions or gels. The highly viscous mixtures that did not show any change in the meniscus after being tilted to an angle of 90º were considered as gels (18).

***Selection of Formulations*:** From the phase diagram showing maximum microemulsion area, a number of microemulsions with different formulae were selected covering the entire range of microemulsion occurrence in the phase diagrams with minimum surfactant and water concentrations and these formulations were subjected to various physical stability tests. The composition of selected microemulsion formulations is given in Table 1.

***Physical Stability Studies*:** Physical stability tests were performed to overcome the problem of metastable formulations. The selected microemulsions were subjected to centrifugation at 5000 rpm for 30 min and the formulations that did not show any phase separation were taken for the heating and cooling cycles. Six cycles between 4ºC (refrigerator temperature) and 45ºC, with storage at each temperature of not less than 48 h, were carried out (19). The formulations that were found stable were subjected to a freeze-thaw cycle test. Formulations were kept in deep freezer (Vestfrost, New Delhi, India) at -20º C for 24 h. Then, microemulsions were removed and kept at room temperature for the next 24 h. Three such cycles were repeated.

Preparation and optimization of Mycophenolate mofetil Loaded Microemulsion Systems: In order to prepare drug loaded microemulsions, mycophenolate mofetil was dissolved in the oily phase containing oleic acid and propylene glycol. Tween80 was solubilized in distilled water. Then the aqueous solution of surfactant was added to the clear oily phase drop by drop under continuous stirring using magnetic stirrer (IKA, Germany) (20). The optimization was carried out by assessing the drug loading capacity of microemulsion systems and the effect of drug loading on globule size of microemulsion (21).

Characterization of Mycophenolate Mofetil Loaded Microemulsion

***Particle Size and Polydispersity Index*:** The average size and polydispersity index of the microemulsion droplets were determined by photon correlation spectroscopy that analyzes the fluctuations in light scattering due to Brownian motion using Malvern Nanosizer ZS (Malvern instruments, UK) (22). Light scattering was monitored at 25°C at a 90° angle.

Refractive Index, Conductivity and pH Measurement: Refractive index was determined for different microemulsion formulations by using Abbe's refractometer (Nirmal International, Delhi, India) at 25°C. Conductivity was measured by

using a digital thermo conductivity meter (Emcee Electronics, Venice, USA). The pH was determined for the optimized microemulsion by using a calibrated digital pH meter (S.D. Fine Chemicals, Mumbai, India) in triplicate at room temperature.

***Zeta Potential Measurements*:** Zeta potential of samples was measured using Beckman Coulter Delsa nanoanalyzer (Beckman Coulter Inc., USA). Samples were diluted with double distilled water prior to analysis and placed in clear disposable zeta cells and the results were recorded. All experiments were performed in triplicate.

Surface Morphology by Transmission Electron Microscopy (TEM): Morphology and structure of the microemulsion were studied using transmission electron microscope (TEM) (Morgagni 268D, FEI, Holland) operating at 70 KV and capable of point-to-point resolution. In order to perform the TEM observations, a drop of microemulsion was placed on carbon-coated copper grid with 2% phosphotungstic acid and observed after drying under electron microscope.

Formulation of Microemulsion Based Gel of MMF: Carbomer 940 was selected as gel matrix for the preparation of microemulsion based hydrogel. 1g of carbopol 940 was dispersed slowly in 100ml of the optimized formulation with the help of an overhead stirrer and the dispersion was then neutralized by dropwise addition of triethanolamine until gelling occurs (10). After neutralization, it was kept in dark for 24 h for complete swelling.

Characterization of Microemulsion Based Gel

***Determination of pH and Spreadability*:** The spreadability of the gel was determined by placing 0.5 g gel within a circle of 1cm diameter premarked on a glass plate over which a second glass plate was placed. A weight of 500 g was placed on the upper glass plate for 5 min and the increase in diameter due to spreading of the gels was noted (23). The pH of the microemulsion based gel was determined using a calibrated pH meter.

***Rheological Studies on the Microemulsion Based Gel*:** The microemulsion based gel system was studied for the rheological behavior at 25±1°C using Anton Paar RheolabQC Rotational Rheometer (Ashland, USA). The viscosity of gel was measured at different shear rates and rheological behavior of the microemulsion gel system was evaluated by constructing rheogram where the shear stress (dyne/cm^2) versus shear rate (s^{-1}) was plotted.

In vitro Skin Permeation Studies: *In vitro* skin permeation studies were performed on a Franz diffusion cell (HEM-100, Harjee Exports Pvt. Ltd., Haryana) with an effective diffusional area of 2.26 cm^2 and receiver chamber capacity of 22.5 ml using rat abdominal skin (10). The full thickness rat skin was excised from the abdominal region, and the hairs were removed. The subcutaneous tissue was removed surgically and dermis side was wiped with isopropyl alcohol to remove adhering fat. The skin was then washed using distilled water and mounted between donor and receptor compartment of Franz diffusion cell, where stratum corneum side faced the donor compartment and dermal side faced the receptor compartment. 1 ml of microemulsion (F2) and microemulsion based gel containing equivalent amount of drug were applied on the surface of rat skin in the donor compartment. Acetate buffer (pH 5.5) was used as receptor media and the temperature in the receptor compartment was maintained at 37±1°C. The receptor phase was stirred at 100rpm using a magnetic stirrer. At predetermined time intervals (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 12, 24 h); 1 ml sample was collected from the receptor compartment and replaced with fresh receptor solution to maintain the sink conditions. The drug solution served as control. All the collected samples were centrifuged and analyzed for MMF content by UV Spectrophotometer at λ_{max} of 250 nm.

The skin permeation rate was calculated from slope of linear plot of cumulative amount

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permeated as a function of time. The flux (J) was calculated by using Eq. (i):

$$J=Dq/dtA \quad (i)$$

Where, J = Flux (μg h^{-1} cm^{-2})

dQ/dt= Slope obtained from linear curve

A= Area of diffusion (cm^2)

The permeability coefficient (K_p) was calculated by dividing J with initial concentration of drug in the donor cell (C_o) by using Eq. (ii):

$$Kp= J/C_o \quad (ii)$$

Release Kinetics : In order to investigate the release mechanism of drug delivery system, the data obtained from *in vitro* drug permeation studies of the optimized microemulsion and microemulsion based hydrogel was fitted into various kinetic models such as zero order, first order, Higuchi and Peppas-Korsemeyer model. Regression analysis was performed for all of these release kinetics models to find out the best fit for drug release from the studied formulations.

In vivo Skin Permeation Studies

For the *in vivo* studies, BALB/c female mice of 8 to 11 week of age were purchased from Indian Institute of Immunology, Jammu. The animals were kept under standard laboratory conditions at temperature of 25±1°C and relative humidity of 55±5%. The animals were housed in polypropylene cages under standard laboratory conditions with free access to food and water *ad libitum.* All the experimental procedures in the animal studies were conducted with prior approval of Institutional Animal Ethical Committee and care of laboratory animals were followed at all the times.

The hair on the dorsal area of the animals were removed carefully using depilatory cream 24 h prior to the experiment and the animals were divided into three groups (Healthy group, Psoriasis control and Group treated with MMF loaded microemulsion based gel). Each group contained six animals.

No formulation was applied to the first group. The other two groups received a daily topical dose of 62.5mg of commercially available imiquimod cream on the dorsal area for 7 consecutive days, translating in a daily dose of 3.125 mg of the active compound (24). In the third group, after induction of psoriatic lesions, the animals were treated with the MMF loaded gel formulation for a period of 7 days. The animals were then sacrificed and skin samples were taken for histopathological studies.

Results and Discussion

***Solubility Studies and Component Selection*:** The results of solubility studies of MMF are shown in Figures 1 and 2. The maximum solubility of MMF was found in oleic acid as compared to other oils. The solubility of MMF in oleic acid was 11.505 ± 0.843 mg/ml, which was highest amongst the oils investigated. In the case of surfactants, highest drug solubility was found in Tween80 (8.882±0.601) mg/ml. The solubility of the drug in propylene glycol was found to be 6.242±0.852 mg/ml. Based on the solubility

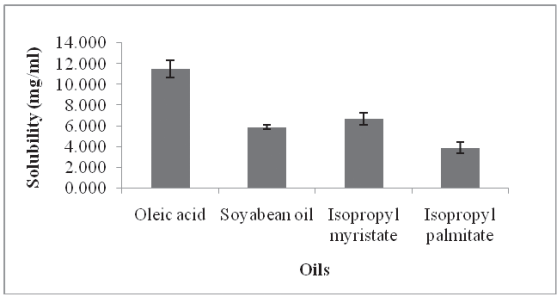

Fig. 1: Solubility of MMF in different oils

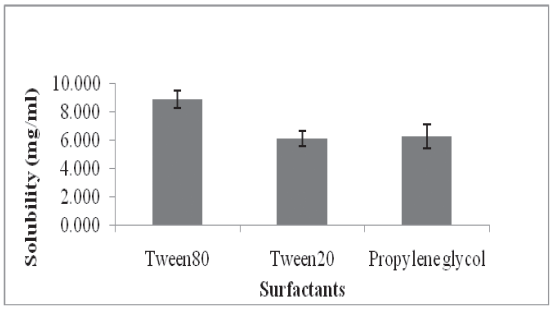

Fig. 2: Solubility of MMF in different surfactants

studies, it was concluded that oleic acid, Tween80 and propylene glycol could be the most appropriate combination for preparation of microemulsion.

***Pseudo-Ternary Phase Diagrams*:** Pseudoternary phase diagrams were constructed separately for each surfactant/ cosurfactant (S_{mix}) ratio i.e. 1:1, 2:1 and 3:1 (Fig. 3) so that microemulsion regions could be identified and microemulsion formulation could be optimized. The maximum water solubilization capacity for the microemulsion systems obtained with S_{mix} ratio of 1:1 was around 50%. It was also observed that within the formed microemulsion zone, the fluidity of microemulsion was reduced with increasing water content. The gel systems were observed when the surfactant concentration was greater than 60% and the water content in the system was in the range of 25-60%. The gel structure was broken down upon further dilution with water before transformation into coarse emulsion. This behavior could be attributed to the fact that the content below 25% is insufficient to hydrate the polyoxyethylene groups of Tween80 which are critical for the swelling of surfactant chains to demonstrate the gel structure. Accordingly, water content more than 60% will increase the distance between the polyethylene groups and destabilize the gel structure leading to breaking of the swollen gel (25).

As the surfactant ratio was increased in the S_{mix} ratio to 2:1, a higher microemulsion region was observed with maximum water solubilizing capacity of 62.2%, perhaps due to further reduction of the interfacial tension and increased fluidity of the interface, thereby increasing entropy of the system. When the S_{mix} ratio of 3:1 was studied, it was found that microemulsion region decreased slightly with maximum percentage of water phase of 45%, which may have been due to decreased concentration of cosurfactant.

***Thermodynamic Stability Studies*:** It is the thermostability that differentiates nano- or microemulsions from emulsions that have kinetic stability and eventually undergoes phase-separation. Microemulsions are formed at a particular concentration of oil, surfactant and water, which makes them stable and not subject to phase separation, creaming or cracking (22). Thus, the formulations were tested for their thermodynamic stability by using centrifugation

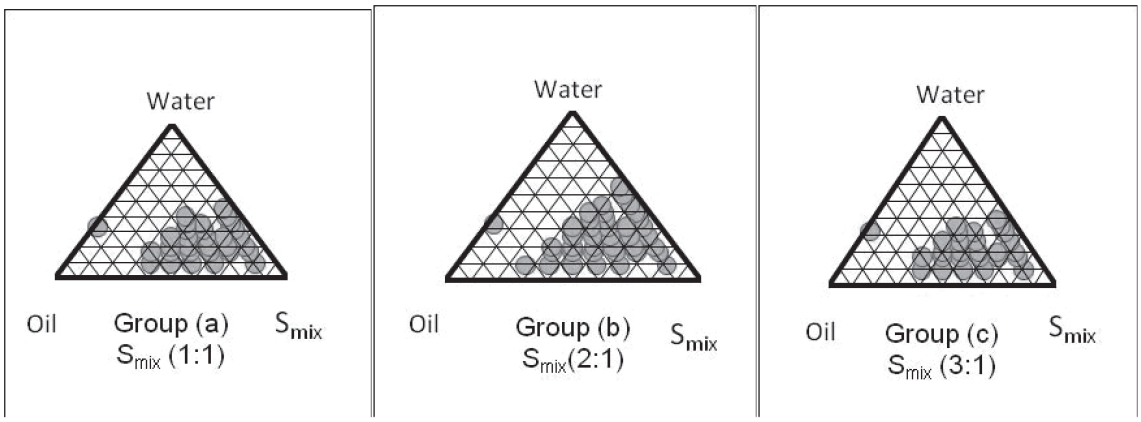

Fig. 3: Pseudoternary phase diagrams developed using the aqueous titration method indicating microemulsion region of oleic acid (oil), Tween 80 (surfactant), Propylene glycol (cosurfactant) at different S_{mix} ratios indicated in Group (a) to Group (c)

studies, a heating-cooling cycle, and a freeze-thaw cycle (22). After performing thermodynamic studies, it was observed that all the selected formulations had shown good stability. No phase separation, creaming or cracking was observed. The results of the studies are shown in Table 2.

***Selection of Formulation*:** From the tested formulations, optimized formulation was selected on the basis of droplet size, polydispersity index, drug loading capacity and the results obtained are described in Table 3. The logic behind selecting these criteria for optimization is that lower droplet size can result in enhanced permeation as well as provide larger surface area for drug release. Polydispersity index (PDI) is the measure of uniformity of the formulation and PDI value less than 1 is desirable (21). Solubility is also an important criterion for the delivery of a poorly water soluble drug. High drug loading capacity ensures large amount of inner phase in microemulsions. The result showed that the smaller droplet size of microemulsion F2 was obtained due to presence of higher concentration of S_{mix} in the microemulsion. The decrease in droplet size with increase in S_{mix} concentration can be attributed to solubilisation of the internal phase within a larger number of surfactant micelles. However in case of formulation F1, containing 6.45% oil and 58.05% of S_{mix}, the average droplet was found to be increased significantly up to 135 nm, which can be attributed to the expansion of oil droplets of microemulsion by increased amount of oil. Based on the results obtained, the formulation F2 was selected for further studies.

***Physicochemical Characterization of Drug Loaded Microemulsion*:** Refractive index is the net value of the components of microemulsion and indicates isotropic nature of formulation. The mean value of refractive index for the formulation F2 was found to be 1.421±0.011. Also the refractive index of drug loaded formulation was determined (1.421±0.006) and compared with that of blank formulation and it was found that there was no significant difference between the values. Therefore, it can be concluded that there were no interactions between microemulsion components and drug.

The specific conductivity of microemulsion F2 was found to be 10^4 µS cm^{-1} and the pH was found to be 5.9±0.04. The zeta potential of the formulation was found to be -34.35±0.051 mV as shown in Fig. 4. The negative values of microemulsions indicated stability of the formulations. The highly negative zeta potential

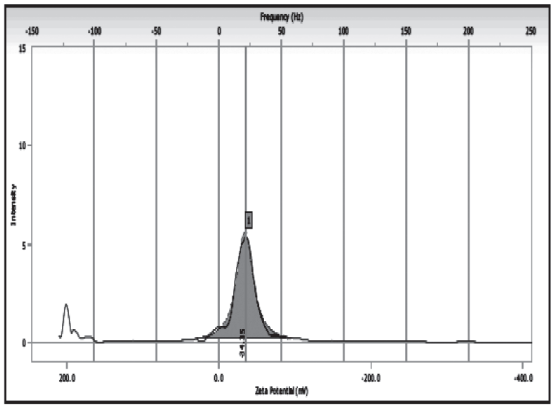

Fig. 4: Zeta potential of MMF loaded microemulsion

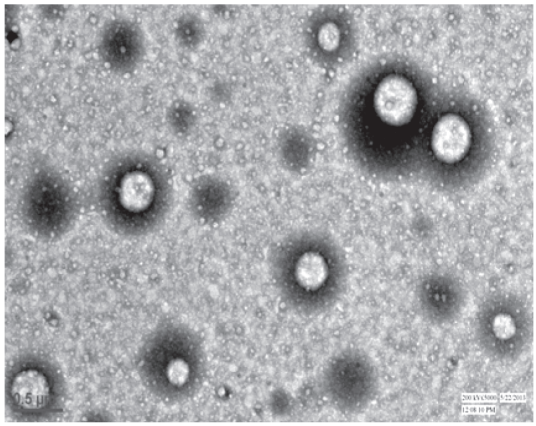

Fig. 5: TEM micrograph of MMF loaded microemulsion

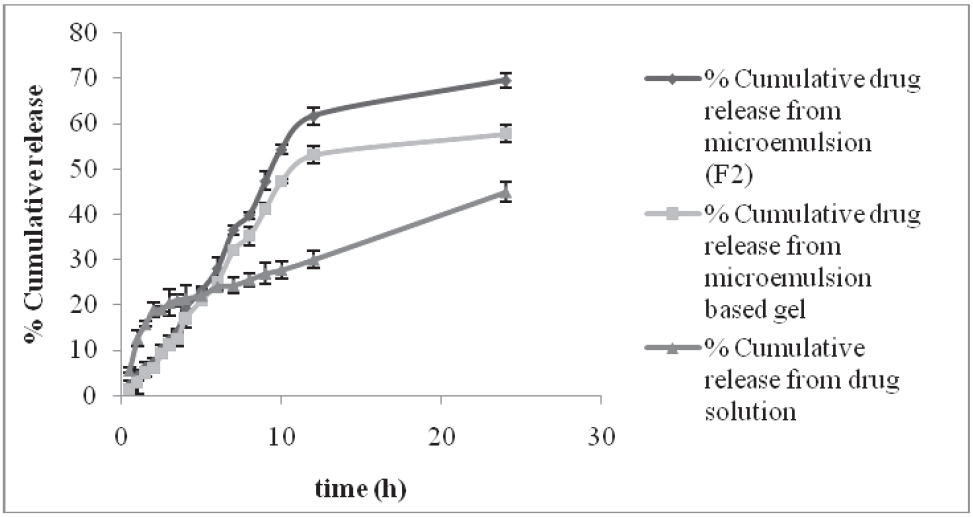

Fig. 6: Cumulative release profile of MMF loaded microemulsion, microemulsion based gel and drug solution.

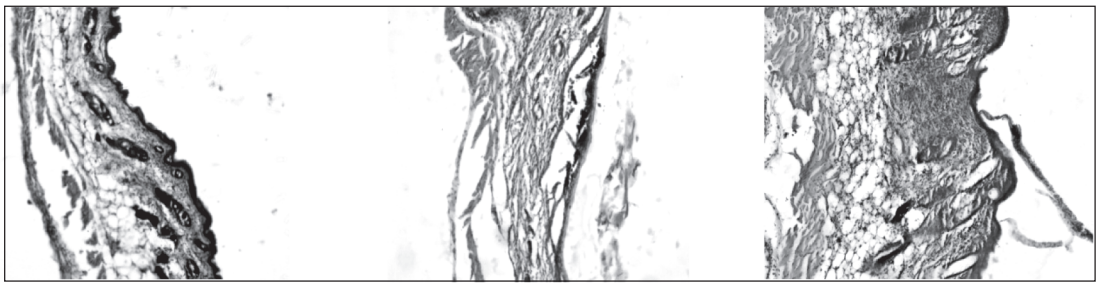

Fig. 7: (A) Photomicrograph of normal skin, (B) Photomicrograph of psoriasis induced skin (C) Photomicrograph of treated skin.

value may be due to the presence of oleic acid at the level of surfactant/cosurfactant film of the microemulsion eliciting an electrostatic repulsion leading to an increase in zeta potential value (26). Surface morphology of the microemulsion formulation F2 was characterized using transmission electron microscopy (Fig. 5) in which the droplets appeared non-aggregated and spherical in shape.

***Hydrogel-thickened Microemulsion*:** Microemulsion gel was prepared using carbopol 940 (1 % w/w). To observe the consistency and homogeneity of the gel, a small quantity of gel was pressed between the thumb and index finger, and it was observed that there were no coarse particles in the optimized gel formulation (17). The spreading of carbopol gel was found to be more uniform and the gel spread in a circular pattern equally on all sides and it almost reached to spreadability diameter of 7.1±0.1 cm upon application of 500 g weight. The pH of the prepared hydrogel thickened microemulsion was found to be 5.5 which is compatible with skin pH.

***Rheological Studies*:** Rheology is an important parameter as it affects the spreadability and adherence of drug. The plot of shear stress versus shear strain was obtained and plot of shear rate versus viscosity was obtained. The

Table 1. Composition of Selected Formulations

Formulation Code	Oleic acid (%v/v)	Tween 80 (%v/v)	Propylene glycol (%v/v)	Total S_{mix} concentration (%v/v)	Water (%v/v)
F1	6.45	38.70	19.35	58.05	35.48
F2	6.06	36.36	18.18	54.54	39.39
F3	5.56	33.33	16.66	49.99	44.44
F4	5	30	15	45	50

Table 2. Results of Thermodynamic Stability Studies

Formulation Code	Heating/Cooling cycle	Centrifuge	Freeze thaw
F1	√	√	√
F2	√	√	√
F3	√	√	√
F4	√	√	√

Table 3. Droplet size, polydispersity values and drug loading capacity of microemulsion formulations

Formulation Code	Droplet Size (nm)*	Polydispersity Index	Drug loading capacity (mg/ml)*
F1	135 nm±11.59	0.091	17.21±1.27
F2	124 nm±10.31	0.504	13.60±0.88
F3	146 nm±15.03	0.128	9.982±0.55
F4	167 nm±18.11	0.141	6.93±00.52

*(mean ± S.D., n = 3)

Table 4. Permeation data of MMF loaded microemulsion and microemulsion based hydrogel

Parameters	Flux (ug/cm^2/h)*	Permeability coefficient x 10^{-3} (cm/h)*
Drug solutions	0.460±0.18	0.338±0.05
MMF loaded microemulsions	1.047±0.35	0.769±0.08
Microemulsion based hydrogels	0.899±0.21	0.661±0.09

*(mean ± S.D., n=3)

Table 5. Kinetic parameters of mycophenolate mofetil released from the microemulsion-based gel

Kinetic models	Correlation (r^2)
Zero order	0.928
First order	0.760
Korsemeyer-Peppas	0.910
Higuchi diffusion	0.549

flow index of the formulation was calculated by plotting graphs of log shear rate versus log shear stress by applying Ostwald-de Waele equation: (27, 28) as given in Table 3.

$$s = Kg^n \qquad \text{(iii)}$$

where, s is shear stress, g is shear rate, K is consistency index and n is flow index. Flow index (dimensionless) is a measure of the deviation from Newtonian behavior (n=1), n < 1 indicates shear thinning (pseudoplastic behavior) and n > 1 shear thickening (dilatant behavior). On the basis of rheological studies performed, the formulated gel was said to follow pseudoplastic behavior (27).

Skin Permeation Studies: To study the influence of formulation on the permeation of mycophenolate mofetil, permeation profile of drug was investigated from drug solutions, MMF loaded microemulsions and microemulsion based hydrogels for a period of 24 h using excised rat abdominal skin on Franz diffusion cell. The drug solutions exhibited only 44.891±2.115%, whereas microemulsion demonstrated 69.524±1.612% of drug permeation in 24 h (Fig. 6). The comparison of cumulative permeation between microemulsion and drug solution showed that MMF loaded microemulsion enhanced the drug permeation significantly ($p< 0.05$). The microemulsion based gel showed slightly lower drug permeation of 57.778±1.812% which may be attributed to slow diffusion of drug through the gel network. Besides providing optimum viscosity to microemulsion for topical application, Carbopol in topical gels was found to provide better adhering of the formulation to skin and delayed drug delivery (18).

Apart from the contribution of oleic acid in enhancing drug permeation in skin by disrupting the fluidity of stratum corneum, the surfactant composition might also be responsible for enhanced permeation from microemulsion. The non-ionic surfactants reportedly emulsify the sebum, thereby enhancing the thermodynamic coefficient of the drug, allowing it to penetrate into cells more effectively (29). The other mechanism depends on the possibility of direct drug transfer from the microemulsion droplet to the stratum corneum. The density of droplets and their surface area are assumed to be high due to the small droplet size and large amount of inner oil phase in the microemulsions. Therefore, droplets settle down to close contact with the skin providing high concentration gradient and improved drug permeation (30).

Permeation parameters like flux (J) and permeability coefficient (K_p) for all the formulations were also calculated and shown in Table 4. The rate of permeation was found to be significantly higher ($p< 0.05$) for microemulsion (1.047±0.109 μg/cm^2/h) and microemulsion based gel system (0.899±0.21 μg/cm^2/h) as compared to drug solution (0.460±0.871 μg/cm^2/h) indicating that microemulsion resulted in considerable improvement in permeation of MMF.

***Release Kinetics*:** To study the release kinetics, various kinetic models including zero-order, first-order, Korsemeyer-Peppas and the Higuchi diffusion model were applied. It was found that *in vitro* release of microemulsion based gel formulation follows zero order (Table 5). It can be attributed to the fact that drug is present in internal phase of microemulsion system. The depletion of drug from the external phase is supplemented by the release of drug from internal phase resulting in the sustained drug delivery from the system (31).

***Histopathological Studies*:** The photo-micrograph of untreated skin (Fig. 7A) showed normal skin with well defined epidermal and dermal layers. Keratin layer was well formed and lied just adjacent to the topmost layer of epidermis and dermis was devoid of any inflammatory cells. Psoriasis induced mice skin closely resembled human plaque type psoriasis with respect to erythema, skin thickening, scaling and epidermal alterations as well as with respect

to inflammatory infiltrate in the epidermal and dermal regions (Fig.7B). Parakeratosis, hyperkeratosis, elongation of rete ridges with supra papillary thinning of epidermis and presence of Munro's abscess in parakeratotic layer were observed as characteristic features of psoriatic lesions were observed (32).

After treatment with the MMF loaded gel formulation (Fig. 7C), keratin layer appeared healthy and showed an increase in the thickness of the epidermis. Hyperkeratosis was not observed and the areas of parakeratosis became significantly reduced in comparison to the control. However, inflammatory infiltrate was observed in dermis and munro's abscess persisted. The results obtained indicated partial recovery of the skin with the application of the formulated MMF loaded microemulsion gel.

Conclusion

In the current study, topical microemulsion gel of mycophenolate mofetil for the treatment of psoriasis was developed which provided enhanced skin permeation of drug and reduced dosing frequency. Different microemulsions were selected from the pseudoternary phase diagrams. The formulation containing 6.06% *v/v* of oleic acid 36.36% *v/v* of Tween80 and 18.18% *v/v* of propylene glycol was considered as optimum formulation having globule size of 124 nm and rate of permeation of 1.047±0.109 µg/cm^2/h. The microemulsion was converted into gel using carbopol 940 (1% *w/v*) so as to improve its viscosity to attain better adherence to skin. The *in vitro* studies revealed that both the microemulsion and microemulsion gel increased the drug permeation through skin as compared to control solution. The *in vivo* studies performed on imiquimod induced skin inflammation on mice skin revealed that the application of the drug loaded microemulsion gel for seven consecutive days lead to complete clearance of hyperkeratotic plaques and a significant reduction in the area of perakeratosis. The results indicated that the formulated gel may be a promising vehicle for the treatment of psoriasis. The future perspective may include elaborate stability and clinical studies for developing commercially viable topical microemulsion formulation of mycophenolate mofetil.

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References

1. Mendonca, C.O. and Burden, A.D. (2003). Current concepts in psoriasis and its treatment. Pharmacology and Therapeutics, 99: 133-147.

2. Katare, O.P., Raza, K., Singh, B. and Dogra, S. (2010). Novel drug delivery systems in topical treatment of psoriasis: Rigors and vigors. Indian Journal of Dermatology, Venereology and Leprology, 76(6): 612-621.

3. Mamta, T., Venkateshwara, Rao, J., Mukkanti, K. and Ramesh, G. (2009). Transdermal drug delivery system for Atomextine hydrochloride- *In vitro* and *ex vivo* evaluation. Current Trends in Biotechnology and Pharmacy, 3(2): 188-196.

4. Paolino, D., Ventura, C.A., Nistico, S., Puglisi, G. and Fresta, M. (2002). Lecithin microemulsions for the topical administration of ketoprofen: percutaneous adsorption through human skin and *in vivo* human skin tolerability. International Journal of Pharmaceutics, 244: 21-31.

5. Shinoda, K., Araki, M., Sadaghiani, A., Khan, A. and Lindman, B. (1991). Lecithin based microemulsions: Phase behavior and microstructure. The Journal of Physical Chemistry, 95(2): 989-993.

6. Lawrence, M.J. and Rees, G.D. (2012). Microemulsion based media as novel drug delivery systems. Advanced Drug Delivery Reviews, 64: 175-193.

7. Lingyun, X., Jinyuan P., Qiyou, C, Qizhi, Y., Huabing C., Huibi, X., Yinsheng, Q. and Xiangliang, Y. (2008). *In vivo* evaluation of the safety of triptolide loaded hydrogel thickened microemulsion. Food and Chemical Toxicology, 46: 3792-3799.

8. Peltola, S., Saarinen-Savolainen, P., Kiesvaara, J., Suhonen, T.M. and Urtti, A. (2003). Microemulsion for topical delivery of estradiol. Journal of Pharmaceutics, 254: 99-107.

9. Baroli, B., Arturo Lopez-Quintela, M., Begona Delgado Charro, M., Fadda, A.M. and Blanco-Mendez, J. (2000). Microemulsions for topical delivery of 8-methoxsalen. Journal of Controlled Release, 69: 209-218.

10. Chang, H., Mou, D., Du, D., Zhu, D., Chang, X., Lui J., Xu, H. and Yang, Y. (2007). Hydrogel- thickened microemulsion for topical administration of drug molecule at an extremely low concentration. International Journal of Pharmaceutical Sciences, 341: 78-84.

11. Shehzad, A., Shaheen, S. and Bokhari, M.A. (2007). Mycophenolate mofetil: a new therapeutic option in dermatology. Journal of Pakistan Association of Dermatologists, 17: 105-111.

12. Hyunkee Park, D.O. and Del Rosso, J.Q. (2011). The emergence of mycophenolate mofetil in dermatology: From its roots in the world of organ transplantation to its versatile role in dermatology treatment room. The Journal of Clinical and Aesthetic Dermatology, 4(1): 18-27

13. Jahn, K., Fisher, A., Neubert, H.H.R. and Wohlrab, J. (2001). Investigation of the penetration behavior of mycophenolate mofetil from a semisolid formulation into human skin *ex vivo*. Journal of Pharmacy and Pharmacology. 53: 1581-1587.

14. Platzer, M., Jahn, K., Neubert, H.H.R. and Wohlrab, J. (2001). Quantification of mycophenolate mofetil in human skin extracts using high performance liquid chromatography-electrospray mass spectrometry, Journal of Chromatography, 755: 355-359.

15. Hashem, F.M., Shaker, D.S., Ghorab, M.K., Nasr, M. and Ismail, A. (2011). Formulation, characterization and clinical evaluation of microemulsion containing clotrimazole for topical delivery. AAPS Pharm. Sci. Tech., 12: 879-886.

16. Verma, S., Gupta, H., Alam, O., Mullick, P., Siddiqui, N. and Khan, S.A. (2009). Spectrophotometric methods for the estimation of mycophenolate mofetil. Journal of Applied Spectroscopy, 76(6): 927-933.

17. Baboota, S., Al-Azaki, A., Kohli, K., Ali, J., Dixit, N. and Shakeel, F. (2007). Development and evaluation of a microemulsion formulation for transdermal delivery of terbinafine. Journal of Pharmaceutical Science and Technology, 61(4): 276-285.

18. Baboota, S., Sharma, S., Alam, M.S., Sahni, J.K., Kumar, A. and Ali, J. (2011). Nanocarrier based hydrogel of betamethasone dipropionate and salicylic acid for the treatment of psoriasis. International Journal of Pharmaceutical Investigation, 1(3): 139-147.

19. Chudasama, A., Patel, V., Nivsarkar, M., Shishoo, C. and Vasu, K. (2011). Investigation of microemulsion system for transdermal delivery of itraconazole. Journal of Advanced Pharmaceutical Technology and Research, 2(1): 30-38.

20. Chen, H., Chang, X., Weng, T., Yang, Y. and Gao, Z. (2004). A study of microemulsion systems for transdermal delivery of triptolide. Journal of Controlled Release, 98: 427-436.

21. El Magharphy, G.M. (2008). Transdermal delivery of hydrocortisone from eucalyptus oil microemulsion: effects of cosurfactants. International Journal of Pharmaceutics, 355: 285-292.

22. Shakeel, F., Baboota, S., Ahuja, A., Ali J., Aqil, M. and Shafiq S. (2007). Nanoemulsions for the transdermal delivery of acceclofenac. AAPS Pharm. Sci. Tech., 8(4): E1-E9.

23. Bachhav, Y.G. and Patravale, V.B. (2009). Microemulsion based vaginal gel of clotrimazole: Formulation, *in vitro* evaluation and stability studies. AAPS Pharm. Sci. Tech., 10: 476-481.

24. Fits, L.V., Mourits, S., Voerman, J.S.A., Kant, M., Boon, L., Laman, J.D., Cornelissin F., Mus, A.M., Florencia, E, Prens, E.P. and Lubberts, E. (2012). Imiquimod - induced psoriasis like skin inflammation in mice is mediated via the IL-23/ IL-17 axis. The Journal of Immunology, 182: 5836-5845.

25. Abdulkarim, M.F., Abdullah, G.Z., Sakeena, M.H.F., Chitneni, M., Yam, M.F., Mahdi, E.S., Ameer, O.Z., Sattar, M.A., Basri, M. and Noor, A.M. (2011). Study of pseudoternary phase diagram behavior and the effects of several tweens and spans on Palm oil esters characteristics. International Journal of Drug Delivery, 3: 95-100.

26. Paolino, D., Venchura, C.A., Nistico, S., Puglisi, G. and Frista, M. (2002). Lecithin microemulsions for the topical administration of ketoprofen: Percutanous absorption through human skin and *in vivo* human skin tolerability. International Journal of Pharmaceutics, 244: 21-31.

27. Shahin, M., Hady, S.A., Hammad, M. and Mortada, N. (2011). Novel jojoba oil based emulsion gel formulations for clotrimazole delivery. AAPS Pharm. Sci. Tech., 12(1): 239-247.

28. Bourret, E., Ratsimbazafy, V., Maury, L. and Brossard, C. (1994). Reological behavior of saturated polyglycolysed glycerides. Journal of Pharmacy and Pharmacology, 46: 538-541.

29. Peltola, S., Saarinen-Savolainen, P., Kiesvaara, J., Suhonen, T.M. and Urtti, A. (2003). Microemulsions for topical delivery of estradiol. International Journal of Pharmaceutics, 254: 99-107.

30. Zhang, J. and Michniak-Kohn, B. (2011). Investigation of microemulsion microstructures and their relationship to transdermal permeation of model drugs: Ketoprofen, lidocaine and caffeine. International Journal of Pharmaceutics, 421: 34-44.

31. Sabale, V. and Vora, S. (2012). Formulation and evaluation of microemulsion based hydrogel for topical delivery. International Journal of Pharmaceutical Investigation, 2(3): 140-149.

32. Krueger, J.G. and Bowcock, A. (2005). Psoriasis pathophysiology: Current concepts of pathogenesis. Annals of Rheumatic Diseases, 64(2): 30-36.

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Cell viability studies and to evaluate anti-cancerous activity of recombinant clone carrying Methionine-γ-lyase (MGL) of *Brevibacterium linens*

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Abstract

Brevibacterium linens is a normal flora present in the whey of curd, which is a rich source of L-Methionine γ-lyase (EC4.4.1.11;MGL), also known as methionase, L-methionine γ-demethiolase. This enzyme can be utilized as a therapeutic agent in treating cancer. The MGL gene was cloned into pRSET-A vector and expressed in *E.coli*DH5α. In light of the importance of MGL of *Brevibacterium linens* in anti-cancerous activity, an attempt was made to analyze its anti-cancerous activity by studying the viability of cancerous cell lines and by studying the toxicity studies on the cancerous cell lines by treating with cloned bacterial extracts. Dried bacterial extract powder was obtained by freeze drying. The percentage viability of HCT 15 and T47D cancer cells carried out by using Trypan blue staining were 71.48%, 79.83% respectively which are most suitable to perform cytotoxicity studies. With the increase in the concentration of the bacterial extract (20 to 120 µl/ml acetone, methanol and ethyl acetate bacterial extracts); the percent viability of cells tends to decrease.

Keywords: *Brevibacterium linens,* Cell viability, Trypan blue staining, MTT assay

Introduction

L-Methionine γ-lyase (EC 4.4.1.11; MGL), also known as methionase, L-methionine γ-demethiolase, and L-methionine methanethiol lyase (deaminating), is a pyridoxal phosphate (PLP)-dependent enzyme that catalyzes the direct conversion of L-methionine to a ketobutyrate, methanethiol, and ammonia by an α,γ -elimination reaction (1). It does not catalyze the conversion of D enantiomers (1-3). L-Methionine γ-lyase is a multifunctional enzyme system since it catalyzes the α,γ - and α,β-elimination reactions of methionine and its derivatives. In addition, the enzyme also catalyzes the β-replacement reactions of sulfur amino acids (2). Many cancer cells have an absolute requirement for plasma methionine, whereas normal cells are relatively resistant to the restriction of exogenous methionine (4). Methionine depletion has a broad spectrum of antitumor activities (5). Under methionine depletion, cancer cells were arrested in the late S-G2 phase due to the pleiotropic effects and underwent apoptosis. Thus, therapeutic exploitation of L-Methionine γ-lyase to deplete plasma methionine has been extensively investigated (6). Growth of various tumors such as Lewis lung carcinoma (7), human colon cancer lines (8), glioblastoma (9), and neuro-blastoma (10) was arrested by MGL. MGL in combination with anticancer drugs such as cisplatin, 5-fluorouracil, nitrosourea, and vincristine displayed synergistic antitumor effects on rodent and human tumors in mouse models (11-14).

Since its discovery in *Escherichia coli* and *Proteus vulgaris* (15), this enzyme has been found in various bacteria and is regarded as a key enzyme in the bacterial metabolism of

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methionine. This enzyme has been partially purified and characterized from *Brevibacterium linens* (16). *B. linens* is a nonmotile, non-spore-forming, non-acid-fast, gram-positive coryneform bacterium normally found on the surfaces of Limburger and other Trappist-type cheeses. This organism tolerates salt concentrations ranging between 8 and 20% and is capable of growing in a broad pH range from 5.5 to 9.5, with an optimum pH of 7.0 (17). In Trappist-type cheeses, *Brevibacteria* depend on *Saccharomyces cerevisiae* to metabolize lactate, which increases the pH of the curd, as well as to produce growth factors that are important for their growth (17). Interest in *B. linens* has focused around its ability to produce high levels of methanethiol. *B. linens* produce various sulfur compounds, including methanethiol, that are thought to be important in Cheddar-like flavor and aroma (18-21). In light of the importance of MGL of *Brevibacterium linens* in anti-cancerous activity, an attempt was made to analyze its anti-cancerous activity by studying the viability of cancerous cell lines and by studying the toxicity studies on the cancerous cell lines by treating with cloned bacterial extracts.

Materials and Methods

Extraction of Bioactive compounds from Brevibacterium linens: The overnight grown cloned *Brevibacterium* MGL gene containing *E.coli* culture broth was centrifuged at 3000 rpm for 15 min to obtain a clear supernatant. Cellular components extracted were successively dissolved in chemical solvents such as acetone, methanol, and ethyl acetate followed by drying of bacterial extracts using vacuum evaporation to obtain the dry extracts. The culture broth was redispensed in 5 ml of Dimethyl sulfoxide (DMSO). The DMSO in the supernatant was then evaporated off and the dried bacterial extract was obtained by freeze drying. Dried bacterial extract powder was collected and stored (22).

***Sample preparation for biological studies*:** From the dried bacterial extract, the bioactive compounds were dissolved in DMSO and transferred to sterile vials of 2 ml capacity; these samples were stored at cold temperature in freezer, protected from light.

***Reagents and Cell Lines*:** Human colorectal adenocarcinoma cancer cell line (HCT 15) and Human breast cancer cells (T47D) were procured from the National center for cell sciences (NCCS), Pune. Cells were maintained in Dulbecco's modified Eagle medium (Hi-Media) supplemented with 10% fetal bovine serum and 100 g/l penicillin/streptomycin and cultured in 5% CO_2 (Thermo scientific) at 37°C. For experimental purpose, cells from exponentially growing culture were used. All experiments were replicated thrice.

***Trypan blue dye exclusion technique*:** Trypan Blue is a blue acid dye that has two azo chromophores group. Trypan blue will not enter into the cell wall of bacterial cells. Trypan Blue is an essential dye, used in estimating the number of viable cells present in a population (23). A cell suspension was made with a fixed volume of cells (e.g. 1ml). Although an aseptic technique is not essential in all stages of this procedure, 50µl of cell suspension was taken and mixed with an equal volume of trypan blue solution with a pipette. After staining with trypan blue solution counting should commence in less than 5 minutes or else the cells will begin to take up the dye. The percentage viability of the HCT 15 and T47D cancer cells were calculated before the treatment with bacterial isolates in trypan blue staining. The percentage of viability of HCT 15 and T47D cancer cells were calculated after treating with the various concentrations of Acetone, Methanol, Ethyl acetate bacterial extracts (20, 40, 60, 80, 100 and 120 µg/ml) in trypan blue staining. It was transferred to a hemocytometer and then the live cells in clear form and dead cell in blue colour were counted. The hemocytometer was placed on the stage of an inverted microscope. Focus was adjusted until a single counting square fills the field.

The number of cells per ml, and the total number of cells were counted using the following formula:

% viability = (live cell count/total cell count) ×100

Microculture tetrazolium (MTT) assay: This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) into an insoluble, coloured formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells (24-26). Evaluation of anti-cancerous activity compound (Acetone, Methanol, Ethyl acetate bacterial extracts) of bacterium, *Brevibacterium linens* was performed using HCT 15, T47D cancer cell line cultures.

HCT 15 and T47D trypsinized cancer cells were seeded onto a 96-well plate at a density of 5000 cells/well in 200 µL of medium for 24 hours. Various concentrations of Acetone, Methanol, Ethyl acetate bacterial extracts (20, 40, 60, 80, 100 and 120 µg/ml), was added to the wells and were incubated for 24 h. A control well without extract was maintained to compare the percent cell viability. After the incubation period 100 µl MTT (5 mg/ml in phosphate buffered saline) was added to each well and incubated for 3 hours in dark. Then MTT was discarded and 150 µl of DMSO was added to each well. The purple colour developed was measured at 570 nm with microplate reader (Bio-Rad). Percent cell viability was calculated as follows.

Percentage of inhibition= 100-(Absorbance of test) / (Absorbance of control) × 100

Growth inhibition were expressed as mean ± SD value of the percentage of absorbance reading from treated cells versus untreated cells

Results and Discussion

***Cancer cell line development and maintenance*:** The cancer cell lines were maintained successfully in laboratory conditions and used for further studies, i.e., Trypan blue staining and MTT assay. Human colorectal adeno carcinoma Cancer cells (HCT 15), Human breast cancer cells (T47D) were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 4.5 g/l glucose and 2mm 1-glutamine and 5% fetal bovine serum (FBS) (growth medium) at 37ºC in 5% CO_2 incubator.

***Trypan blue staining*:** Cell lines were free from bacterial and fungal contamination, percentage of cell viability of cell lines were carried out by using Trypan blue staining, The percentage of

Table 1. Percentage of cell viability of cell lines before Trypan blue staining

Cell line	% viability	Live cell count	Total cell count	PH
T47D	79.83	1.932×10^5	2.42×10^5	7.5
HCT 15	71.48	1.88×10^5	2.63×10^5	6.9

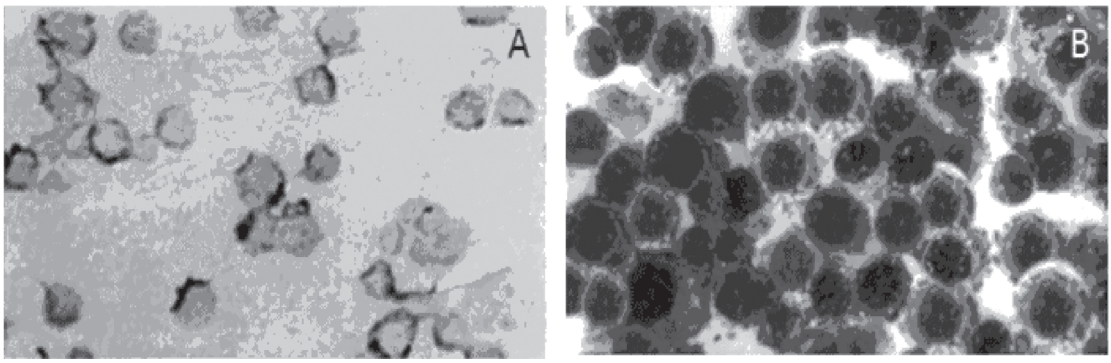

Fig.1: Trypan blue staining (A: Live cells, B: Dead cells)

Cell viability studies and to evaluate anti-cancerous

viability of the HCT 15 and T47D cancer cells before the treatment with bacterial extract in trypan blue staining, showed viability of HCT 15 cell line and T47D cell line as 79.83% and 71.48% respectively, which are most suitable to perform cytotoxicity studies (Table.1; Fig.1). The percentage viability of HCT 15 and T47D cancer cells after treating with bacterial extract (i.e., with acetone, methanol and ethyl acetate bacterial extracts) at different concentrations in trypan blue staining. With the increase in the concentration of the bacterial extract (20 to 120 µl/ml), the percentage of viability of cells tends to be decreased.

***MTT Assay with recombinant bacterial extracts*:** In our present study the impact of recombinant bacterial extracts of Acetone, Methanol and Ethyl acetate on the growth of HCT 15 and T47D cancerous cell line was examined by performing MTT assay. After the treatment of cell lines with various concentrations of extracts, the results from MTT assay shows that there is an exponential increase in the growth inhibition as the concentration is increased. With the increase in the concentration of the bacterial extract from 20 to 120 µl/ml, the percent inhibition for HCT 15 cancer cell line increased from 22.83 to 65.16 % in Acetone bacterial extract, 32.64% to 76.75 % in Methanolic bacterial extract and 36.43 % to 90.05 % in Ethyl acetate bacterial extract, which means they induced cell arrest to inhibit the growth of the HCT 15 cancer cells. In the same way, in case of T47D, as the concentration of Acetone, Methanol and Ethyl acetate bacterial extracts increased from 20 to 120 µl/ml, the percent inhibition of T47D cancer cell growth increased from 18.43 % to 60.76 % in Acetone bacterial extract, 24.88 % to 73.01 % in Methanol bacterial extract and 32.82 % to 83.14 % in Ethyl acetate bacterial extract (Table 2 & 3).

Table 2. Percentage of HCT 15 cell inhibition at various concentrations with recombinant extracts of Bacteria

Concentration (µg/ml)	Percentage of HCT 15 cell inhibition		
	Acetone Bacterial extract	Methanol Bacterial extract	Ethyl acetate Bacterial extract
20	29.38	32.64	36.43
40	35.31	41.81	44.96
60	41.66	50.75	60.52
80	49.47	61.27	67.92
100	57.3	66.08	73.84
120	72.95	76.75	90.53

Table 3. Percentage of T47D cell inhibition at various concentrations with recombinant extracts of Bacteria

Concentration (µg/ml)	Percentage of T47D cell inhibition		
	Acetone Bacterial extract	Methanol Bacterial extract	Ethyl acetate Bacterial extract
20	22.83	24.88	32.82
40	32.02	33.79	41.92
60	34.84	39.12	50.21
80	44.37	50.94	60.79
100	61.18	63.73	68.12
120	65.16	73.01	83.14

Conclusion

The percentage of viability of HCT 15 and T47D cancer cells free from bacterial and fungal contamination carried out by using Trypan blue staining were 71.48%, 79.83% respectively which were most suitable to perform cytotoxicity studies. With the increase in the concentration of the bacterial extract (20 to 120 µl/ml acetone, methanol and ethyl acetate bacterial extracts), the percentage of viability of cells tends to be decreased. By subjecting the cell lines to MTT assay, it was concluded that ethyl acetate bacterial extract showed maximum inhibition of cancerous cell growth.

References

1. Tanaka, H., Esaki, N., and Soda, K. (1985) A versatile bacterial enzyme: L-methionine c-lyase. Enzyme. Microb. Technol. 7, 530-537.

2. Tanaka, H., Esaki, N., and Soda, K. (1983) Properties of L-methionine gamma-lyase from *Pseudomonas ovalis*. Biochemistry. 16, 100-106.

3. Tanaka, H., Esaki, N., Tammamoto, T., and Soda, K. (1976) Purification and properties of methionine g-lyase from *Pseudomonas ovalis*. FEBS Lett. 66, 307-311.

4. Cellarier, E., Durando, X., Vasson, M. P., Farges, M. C., Demiden, A., Maurizis, J. C., Madelmont, J. C., and Chollet, P. (2003) Methionine dependency and cancer treatment. Cancer. Treat. Rev. 29, 489-499.

5. Kokkinakis, D. M. (2006) Methionine-stress: a pleiotropic approach in enhancing the efficacy of chemotherapy. Cancer. Lett. 233, 195-207.

6. Yoshioka, T., Wada, T., Uchida, N., Maki, H., Yoshida, H., Ide, N., Kasai, H., Hojo, K., Shono, K., Maekawa, R., Yagi, S., Hoffman, R. M., and Sugita, K. (1998) Anticancer efficacy in vivo and in vitro, synergy with 5-fluorouracil, and safety of recombinant methioninase. Cancer. Res. 58, 2583-2587.

7. Tan, Y., Sun, X., Xu, M., Tan, X., Sasson, A., Rashidi, B., Han, Q., Tan, X., Wang, X., An, Z., Sun, F. X., and Hoffman, R. M. (1999) Efficacy of recombinant methioninase in combination with cisplatin on human colon tumors in nude mice. Clin. Cancer. Res. 5, 2157-2163.

8. Kokkinakis, D. M., Hoffman, R. M., Frenkel, E. P., Wick, J. B., Han, Q., Xu, M., Tan, Y., and Schold, S. C. (2001) Synergy between methionine stress and chemotherapy in the treatment of brain tumor xenografts in athymic mice. Cancer. Res. 61, 4017-4023.

9. Hu, J., and Cheung, N. K. (2009) Methionine depletion with recombinant methioninase: in vitro and in vivo efficacy against neuroblastoma and its synergism with chemotherapeutic drugs. Int. J. Cancer. 124, 1700-1706.

10. Miki, K., Xu, M., Gupta, A., Ba, Y., Tan, Y., Al-Refaie, W., Bouvet, M., Makuuchi, M., Moossa, A. R., and Hoffman, R. M. (2001) Methioninase cancer gene therapy with selenomethionine as suicide prodrug substrate. Cancer. Res. 61, 6805-6810.

11. Spallholz, J. E., Palace, V. P., and Reid, T. W. (2004) Methioninase and selenomethionine but not Se-methylselenocysteine generate methylselenol and superoxide in an *in vitro* chemiluminescent assay: implications for the nutritional carcinostatic activity of selenoamino acids. Biochem. Pharmacol. 67, 547–554.

12. Yang, Z., Wang, J., Yoshioka, T., Li, B., Lu, Q., Li, S., Sun, X., Tan, Y., Yagi, S., Frenkel, E. P., and Hoffman, R. M. (2004). Pharmacokinetics, methionine depletion, and antigenicity of recombinant methioninase in primates. Clin. Cancer. Res. 10, 2131-2138.

13. Sun, X., Yang, Z., Li, S., Tan, Y., Zhang, N., Wang, X., Yagi, S., Yoshioka, T., Takimoto, A., Mitsushima, K., Suginaka, A., Frenkel, E. P., and Hoffman, R. M. (2003) In vivo efficacy of recombinant methioninase is enhanced by the combination of polyethylene glycol conjuga-tion and pyridoxal 5^0-phosphate supplementation. Cancer. Res. 63, 8377-8383.

14. Takakura, T., Takimoto, A., Notsu, Y., Yoshida, H., Ito, T., Nagatome, H., Ohno, M., Kobayashi, Y., Yoshioka, T., Inagaki, K., Yagi, S., Hoff-man, R. M., and Esaki, N. (2006) Physicochemical and pharmacokinetic characterization of highly potent recombinant L-methionine gamma-lyase conjugated with polyethylene glycol as an antitumor agent. Cancer. Res. 66, 2807-2814.

15. Onitake, J. (1938) On the formation of methyl mercaptan from L-cystine and L-methionine by bacteria. J. Osaka Med. Assoc. 37, 263-270.

16. Collin, J. C. and Law, B. A. (1989) Isolation and characterization of the L-methionine-g-demethiolase from *Brevibacterium linens* NCDO 739. Sci.Aliments. 9, 805-812.

17. Purko, N. O., Nelson, N. O., and Wood, W. A. (1951) The associative action between certain yeasts and *Bacterium linens*. J. Dairy Sci. 34,699-705.

18. Boyaval, P., and Desmazeaud, M. J. (1983) Le point des connaissances sur *Brevibacterium linens*. Lait. 63, 187-216.

19. Ferchichi, M., Hemme D. and Nardi M. (1986) Induction of methanethiol production by *Brevibacterium linens* CNRZ 918. J. Gen. Microbiol. 132, 3075-3082.

20. Hemme, D., Bouillanne, C., Metro, F., and Desmazeard M. J. (1982) Microbial catabolism of amino acids during cheese ripening. Sci. Aliments. 2, 113-123.

21. Sharpe, M. E., Law, B. A., Phillips, B. A. and Pitcher, D. G. (1977) Methanethiol production by coryneform bacteria: strains from dairy and human skin sources and *Brevibacterium linens*. J. Gen. Microbiol. 101, 345-349.

22. Mohanta T. K., Patra J. K., Rath S. K., Pal D. K., and Thatoi H. N. (2007) Evaluation of antimicrobial activity and phytochemical screening of oils and nuts of *Semicarpus anacardium*, Scientific Research and Assay. 11, 486-490.

23. Phillips, H. J., and Terryberry, J. E. (1957) Counting actively metabolizing tissue cultured cells. Cell Research. 13, 341-347.

24. Mosmann, T. (1983) Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of Immunology Methods. 65, 55-63.

25. Wilson, A. P. (2000) Cytotoxicity and Viability Assays in Animal Cell Culture: A Practical Approach. 3rd ed, Oxford University Press: Oxford Vol. 1.

26. Masters, R. W. (2000) Animal cell culture: Cytotoxicity and viability assays. 3rd ed. p. 207.

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Studies on Pectinase Production by *Enterobacter* sp. using Mango Fruit Processing Industrial Waste as Whole and Sole Carbon Source

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Abstract:

Mango Fruit Processing Industrial Waste (MIW) is a pectin rich carbon source utilized as substrate for the production of pectinase from *Enterobacter* sp. in submerged fermentation process. Eight bacterial strains were isolated and screened (Pectin Clear Zone technique) for their ability to produce pectinase. Among them, *Enterobacter* sp. has given highest PCZ value of 34 mm. In secondary screening, pectinase production by *Enterobacter* sp. has been studied under the suitable fermentation conditions such as temperature- 38 °C, pH- 6.0, inoculum-size- 0.6 ml/100 ml, incubation- 96 hrs, substrate concentration- 0.6 g/100 ml, carbon source- fructose (1 %), Riboflavin (1 %). The effect of different amino acids, vitamins also studied. Under these suitable conditions the highest pectinase activity of 82.647 U/ml observed. These results suggesting that, the production of pectinase in large scale using MIW is a low cost method with high value product i.e., pectinase. The utilization of this waste for pectinase production will also control the environmental pollution.

Key words: Mango Fruit Processing Industrial Waste (MIW), *Enterobacter* sp. Solid State Fermentation (SSF), Submerged Fermentation (SmF), Pectinase.

Introduction

Mango (*Mangifera indica L.*) is an important fruit crop of India. India stands at top position in the mango production in the world, with its 12749.8 million tons of production per year (1, 2). Many number of mango fruit processing industries have established in Andhra Pradesh as it is contributing major amount of mangos to an average production of India. A huge amount of waste have been generating, while processing the mango fruit and causing severe environmental pollution due to its microbial contamination. In order to control this, the mango fruits processing industrial waste was utilized for the production of commercially valuable products. The microbial transformation of agro industrial wastes had produced various valuable products like bio-gas, ethanol, enzymes, volatile flavoring compounds, fatty acids and microbial biomass (3). Similarly, dried mango industrial waste also used for production of pectinase, as it contains an appreciable amount of pectin and carbohydrates, proteins, the fat content, however, is low (4). Pectin acts as the inducer for the production of pectinolytic enzymes by both submerged and solid state fermentations. But submerged fermentation requires high volumes of water, continuous agitation and generates lot of effluents (5). Earlier many bacteria like *Bacillus, Aeromonas, and Lacto bacillus* etc.., used for the production of pectinase. It is well known that as compared to intracellular enzymes, the extra cellular enzymes are easier to be extracted. Pectin itself can be extracted from mango industrial waste (mainly from peels) as a commercially important by-product. Earlier some other wastes like apple pomace, mango waste, orange waste, other fruit and vegetable industrial wastes and different agro-industrial wastes were used for the production of enzymes such as

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pectinase, cellulase, α-amylase, esterase and peroxidase (6, 7). Pectinases are group of enzymes that attack pectin and de-polymerise it by hydrolysis and transelimination as well as by de-esterification reactions, which hydrolyses the ester bond between carboxyl and methyl groups of pectin (8). These enzymes act on pectin, a class of complex polysaccharides found in the cell wall of higher plants and cementing material for the cellulose network (9). The present investigation was undertaken to produce pectinase by the isolated bacterial strain *Enterobacter* sp. for the conversion of mango fruit processing industrial waste into humus via microbial transformation in submerged fermentation process.

Materials and Methods

Sample Collection: The processed waste produced from mango fruit processing industries (wet, dry and soil) were collected in sterile polythene covers from different MIW yards around Chittoor district, A.P., India and stored at 4 °C for further use. This waste (dried and powdered) used as carbon source/Nutrition for isolates.

Isolation and Identification of Bacteria *:* The bacterial strains were isolated by serial dilution of 1.0 g MIW. The pure cultures of isolates were made by Streak-plate method on nutrient agar media slants. The isolates were identified by gram's staining, morphological and biochemical characterization of colonies on agar slants described in K R Aneja lab manual (10).

Primary Screening (Screening test-I)*:* Modified Czapec dox's broth used as production medium and screening assay has done on nutrient agar medium supplemented with 4 % Pectin.

Pectinase Production Medium (PPM)*:* This medium consists of part (A) and part (B). Part (A) contained (g/l): $NaNO_3$ - 2.0; KH_2PO_4 - 1.0; KCl - 0.5; $MgSO_4$.$7H_2O$ - 0.5; Yeast extract - 1.0. These contents were dissolved in 40 ml distilled water. The pH was adjusted to pH 7.0 by NaOH (5 %, w/v). Part (B) contained (g/l): Pectin, 5.0, dissolved in 10 ml. of distilled water. The two parts (A) and (B) were mixed and sterilized. Then inoculated with bacterial isolates and incubated at 37 °C for 96 hrs, then assayed for pectinase activity.

Pectinase Activity Assay *:* Nutrient agar medium with 4 % Pectin, pH 7.0 was used as assay medium. Plates of the same size poured with equal amounts of sterilized assay medium. Upon solidification three wells were made, each well inoculated with 0.1 ml of culture filtrate. These plates incubated at 37 °C for 2-4 days and then plates flooded with Hexadecyl Trimethyl Ammonium Bromide (HTAB) solution, clearing zones of the medium investigated and taken as the criteria for determining the pectinase productivity.

Secondary Screening (Screening test-II)*:* In secondary screening, pectinase produced in submerged fermentation and its activity assayed by DNS method. Mango fruit processing industrial waste powder Basal medium was used as production medium (MIWP-BM).

MIWP-Basal Medium: The basal medium (BM) was prepared according to Vincent method. It contained the following (g/l): $NaNO_3$ - 2.0; K_2HPO_4 - 0.5; KCl - 0.5 and yeast extract 1 %. These were dissolved in citrate phosphate buffer at pH- 7.0 and supplemented with MIW powder (4 % w/v) separately. Then pH of this medium was adjusted to 7.0 and sterilized at 121°C. This medium was inoculated with 0.5 ml of overnight culture broth of *Enterobacter* sp. incubated at 37 ºC for 6 days. Then the pectinase activity was assayed for every 24 hrs.

Pectinase Assay**:** The pectinase activity assayed by DNS method (11). 0.5 ml of culture filtrate was used as enzyme source and 0.5 ml of 1% pectin was used as substrate. One unit (1U) of enzyme activity is equal to the 1 µMol of reducing sugars released, measured in terms of D-galacturonic acid, produced as a result of enzyme-substrate reaction.

Factors influencing the pectinase production:

***Effect of Temperature**:* The *Enterobacter* sp. grown in MIWP-Basal Medium at different temperatures viz. 18 °C, 28 °C, 38 °C, and 48 ÚC, for about 6 days for the production of pectinase. Measurement of pectinase productivity was performed for every 24 hrs at 540 nm by spectrophotometer.

***Effect of pH**:* The pH of the production medium of different culture flasks was adjusted to 5.0, 6.0, 7.0, 8.0, by using 0.1 N NaOH and 0.1 N HCl. Then flasks were inoculated with *Enterobacter* sp. and incubated at 38°C. Then the Pectinase activity was assayed.

***Effect of Substrate concentration**:* Different concentrations of substrate (g/100 ml flask, (w/v)) 0.2, 0.4, 0.6, and 0.8, added in production medium, pH adjusted to 6.0. Then flasks were inoculated with *Enterobacter* sp. and incubated at 38 °C. Then the Pectinase activity was assayed.

***Effect of Inoculum-size**:* The overnight broth culture of *Enterobacter* sp. was used as inoculum. The inoculum sizes (ml/100 ml) 0.2, 0.4, 0.6, and 0.8 inoculated in to production medium containing 0.6 g of substrate at pH 6.0. Then the inoculated flasks were incubated at 38 °C and Pectinase activity was assayed.

***Effect of different Incubation periods**:* Under the suitable culture conditions such as pH-6.0, substrate concentration (0.6 g) production medium was inoculated with 0.6 ml of *Enterobacter* sp. culture broth and incubated at 38 °C. Then the pectinase activity was measured every day at 2 hrs, 4 hrs, 8 hrs, and 16 hrs of time intervals.

***Effect of Carbon source**:* Different external carbon sources were introduced into the production medium at an equimolecular amount located at 1 % (w/v) sucrose. Parallel experiment was made with no sugar as a control. The carbon sources, dextrose, fructose, and lactose and mannose and pectin were introduced at the level of 1 % (w/v). Under the above mentioned (in 2.4.5) cultural conditions the culture flasks were incubated for 52 hrs and Pectinase activity was assayed.

***Effect of Nitrogen source**:* Production medium was supplemented with different nitrogen sources at an equimolecular amount of nitrogen that present in sodium nitrate (0.2 %, w/v) in basal medium. The applied nitrogen sources ammonium oxalate, potassium nitrate, peptone, urea introduced as organic nitrogen source at the level of 1 % and the control was devoid from any nitrogen source. All the experiments were carried out pectinase activity was assayed.

***Effect of Amino acids**:* The production medium was added at an equimolecular amount of nitrogen located in the best inorganic nitrogen source for the pectinase productivity. This experiment was controlled by performing of parallel one containing the original nitrogen source i.e., sodium nitrate and was devoid of any amino acid. The supplemented amino acids: alanine, glycine, phenyl alanine, and methionine. All the experiments were carried out and pectinase activity was assayed.

***Effect of Vitamins**:* Different vitamins are ascorbic acid, riboflavin, vitamin-B6 and vitamin-E added separately to flasks containing the pectinase production medium, while the control applied free from any vitamin. All the experiments were carried out and assayed pectinase activity was assayed.

Results and Discussion

Sample collection: Thirty mango fruit processing industrial waste samples collected from ten different mango fruit processing industries around the Chittoor district. They were used as a source for the isolation of pectinase producing bacterial strains. Earlier the wastes like Orange peel, Citrus peel and Potato peel were also used as a source for isolating pectinolytic microorganisms (12).

Isolation and identification of bacteria: Eight bacterial strains were isolated and pure cultured on nutrient agar slants. Based on the results of

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morphological and biochemical characteristics (Table-1 and 2), the isolated bacterial strains were identified as *Enterobacter* sp. *Serratia* sp. *Enterobacterium* sp., *Providencia* sp., *Raoutlella* sp., *Pectobacterium* sp., *Entero-bacteriaceae bacterium* sp., *Morganella* sp. (Table-3). Similarly bacterial isolates such as *Bacillus firmus*-I-4071, *B. firmus*-I-10104 and *Bacillus laterosporus*-I-107 were reported from agro and fruit processing industrial wastes (7, 12 and 13).

Primary screening (Screening test-I): All eight bacterial strains considered as good pectinase producers with their PCZ values of pectinase plate assay (Table-4). One of them, *Enterobacter* sp. has given highest pectinase productivity with its PCZ value of 34 mm (Fig. 1). The PCZ value of *Enterobacter* sp. strain was very much similar with that of 3 bacterial isolates; 4071, 107 and 10104 with pectin clear zones of 32, 34 and 34 mm respectively (by using *Solanum tuberosum* peels as substrate) (14).

Secondary screening (Screening test-II): Since 1940s, pectinases have been used in several conventional industrial processes, such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial wastewater, purification of viruses, in making of paper, for increasing filtration efficiency and clarification of fruit juices, in wood preservation and used in maceration, liquefaction and extraction of vegetable tissues (15). For this reason, *Enterobacter* sp. have examined for its ability to utilize mango fruit processing industrial waste as substrate. The Physico-chemical characteristics

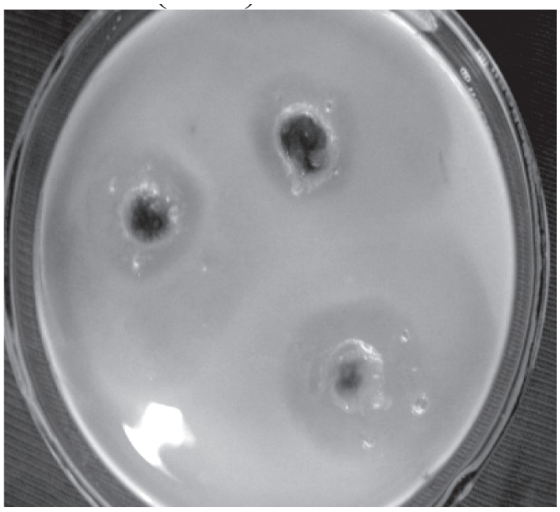


Fig. 1: Pectin Clear zones of *Enterobacter* sp. PCZ- Pectin clear zone, mm- Millimeter;

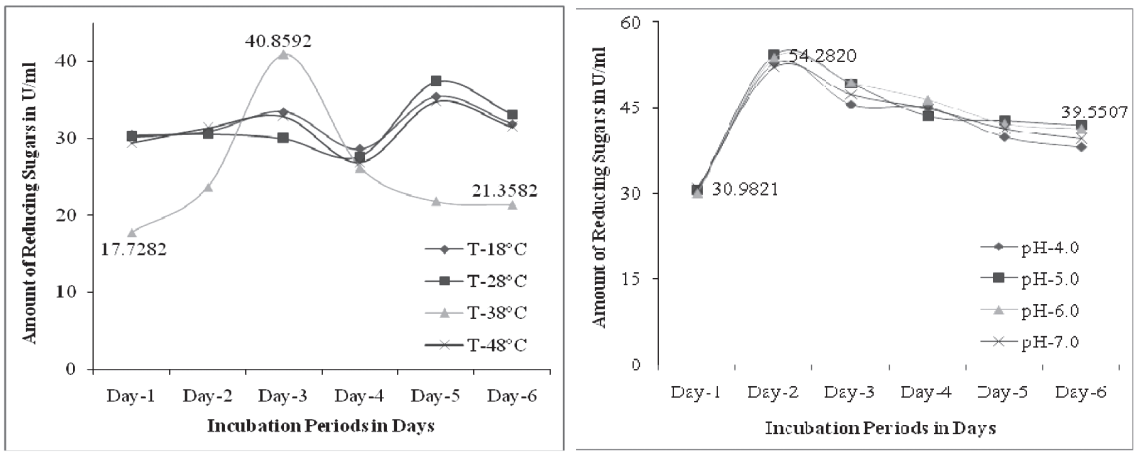
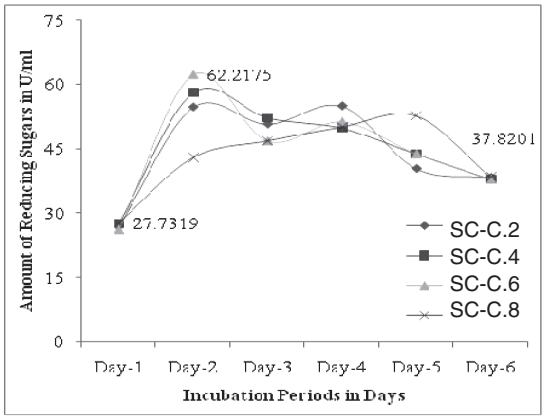


Fig. 2 and 3. Effect of *Temperature (°C)* and *pH* on the pectinase production by *Enterobacter* sp.



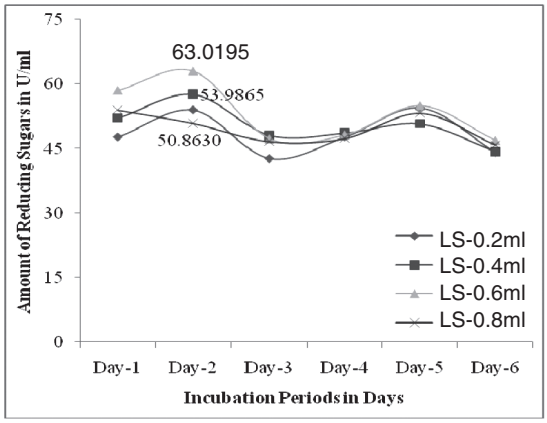
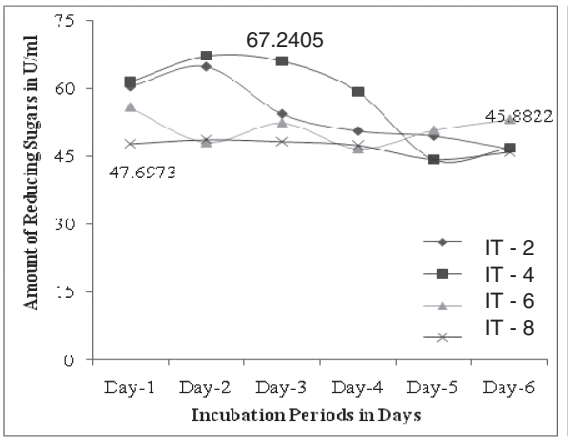


Fig. 4 and 5. Effect of *Substrate concentration (g/100ml)* and *Inoculum size (ml)* on the pectinase production by *Enterobacter* sp.



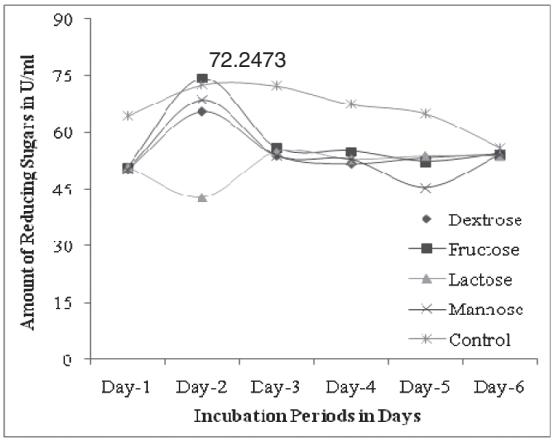
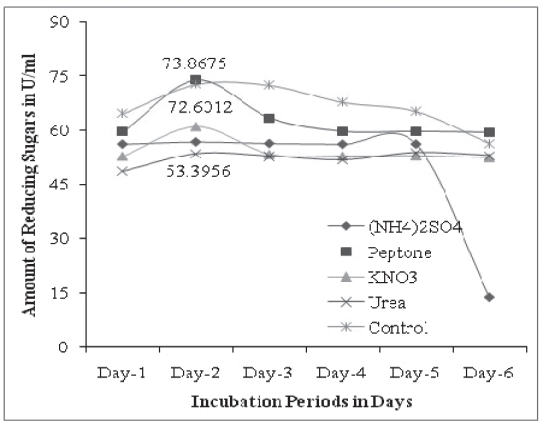


Figure 6 and 7: Effect of *Incubation periods (Hours) and Carbon Source (1%)* on the pectinase production by *Enterobacter* sp. Dex- Dextrose, Fru-Fructose, Lac- Lactose, Man- Mannose, Con- Control *3.4.4. Effect of Nitrogen Source (1%) and Amino acids (1%):*



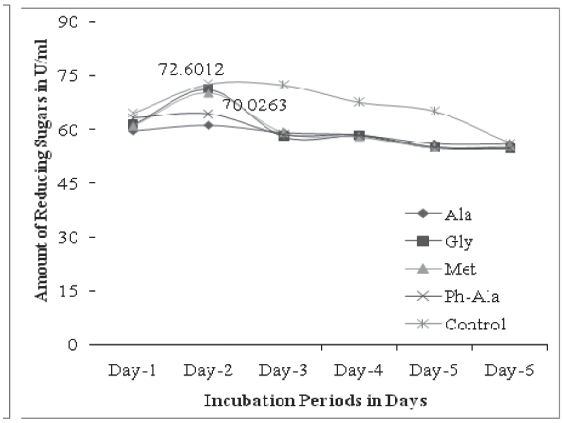


Fig. 8 and 9: Effect of *Nitrogen Source 1%* and *Amino acids 1%* on the pectinase production by *Enterobacter* sp. Ala-Alanine, Gly-Glysine, Phe-Phenyl alanine, Met-Methionine

of the fermentation medium play an important role in pectinase production from *Enterobacter* sp. (Fig. 2). The highest pectinase production was observed at 38°C, upon increasing or decreasing the temperature the pectinase production was decreased. Similarly pH of the medium, incubation time, substrate concentration and inoculum size will also affect the pectinase production. It was observed that from Fig. 2 to Fig. 6, shows that the optimal cultural conditions increased the pectinase activity 40.859 U/ml to 67.24 U/ml. Similarly the high pectinase production was observed at 72 hrs of incubation at 35°C with the initial pH of 6.5 using *Bacillus* sp. MFW7 (16). The presence of external carbon source (Fructose) and added vitamins (Riboflavin) in the production medium increased the pectinase productivity to 82.647 U/ml while the presence of external nitrogen source and added amino acids in production medium decreased the pectinase productivity to 71.123 U/ml [(Peptone) (Fig. 7 to Fig. 10). In presence of 0.5% of pectin (carbon source) the FW2 isolate showed highest activity of 22 U/ml (17), similarly Lactose in combination with peptone supported maximum pectinase production by *Bacillus* sp. MFW7 (18). The pectinase activity of *Enterobacter* sp. 82.647 U/ml was observed under the standardized cultural conditions is very much higher than the activity of reference bacterial strain OS-IV (29.1 U/ml) isolated from soil of a plum tree orchard and agro-industrial waste (19, 20). This shows that the chemical composition of the medium will also play an important role in pectinase production from microbes. The economic and ecological function of pectinase enzymes in industries is gaining much attention with the need of highly productive strains of microorganisms to reduce production cost. Production of pectinase by *Enterobacter* sp. using MIW as substrate in submerged fermentation is a very low cost method. In this process 25 g of purified pectinase can be extracted using 1000 g of MIW. The cost for whole fermentation and purification processes of pectinase is half of the market value of 25 g pectinase ($295.0) (21). This shows that the utilization of freely available MIW for pectinase production by *Enterobacter* sp. is more suitable for industrial large scale production.

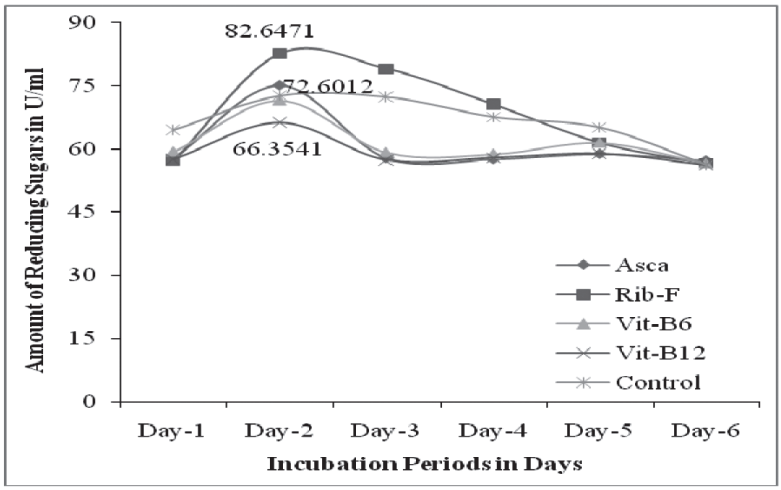


Fig. 10. Effect of *vitamins (1%)* on the pectinase production by *Enterobacter* sp. Aa- Ascarbic acid, Rib- Riboflavin, V- B_6-Vitamin- B_6, V-E- Vitamin- E

Table 1. Morphological characters of isolated bacterial pure cultures:

Character Isolate	Colony	Tempe-rature	Growth	Form	Margin	Elevation staining	Density	Gram's	Identified as
PSTB1	White	35ÚC	Agar(+)	Rhizoid	Filamentous	Flat	Translucent	Rod+	*Bacilli (+)*
PSTB2	White	35ÚC	Agar(+)	Circular	Raised	Flat	Translucent	Round +	*Cocci (+)*
PSTB3	White	35ÚC	Agar(+)	Irregular	Filamentous	Flat	Translucent	Rod chains-	*Strepto bacilli (-)*
PSTB4	White	35ÚC	Agar(+)	Irregular	Lobate	Flat	Translucent	Round bunch +	*Staphylo coccus(-)*
PSTB5	White	35ÚC	Agar(+)	Rhizoid	Lobate	Raised	Translucent	Round bunch -	*Staphylococcus(-)*
PSTB6	White	35ÚC	Agar(+)	Irregular	Filamentous	Entire	Translucent	Round chains+	*Diplo coccus (+)*
PSTB7	White	35ÚC	Agar(+)	Irregular	Lobate	Flat	Translucent	Round -	*Cocci (-)*
PSTB8	Pink	35ÚC	Agar(+)	Irregular	Filamentous	Flat	Translucent	Round bunch +	*Staphylo coccus(+)*

Note: '+'= Positive, '-'= Negative

Table 2. Bio-Chemical charecteristics of isolated Bacterial Strains:

IsolateTest name	***PSTB1***	***PSTB2***	***PSTB3***	***PSTB4***	***PSTB5***	***PSTB6***	***PSTB7***	***PSTB8***
Casein Hydrolysis	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Lactose Fermentation	Negative	Positive	Positive	Negative	Positive	Positive	Positive	Negative
Dextrose Fermentation	Positive	Positive	Positive	Positive	Positive	Negative	Positive	Positive
Sucrose Fermentation	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive
H2S Production	Positive	Positive	Positive	Negative	Negative	Positive	Positive	Positive
NO3 Reduction	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Indole Production	Positive	Positive	Negative	Negative	Negative	Positive	Positive	Positive
MR Reaction	Positive	Negative	Positive	Positive	Positive	Negative	Positive	Positive
VP test	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Citrate utilization	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Urease activity	Negative	Negative	Positive	Negative	Positive	Negative	Negative	Negative
Catalase activity	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Oxidase activity	Negative	Positive	Negative	Negative	Positive	Positive	Positive	Positive
Gelatin Liquification	Negative	Positive	Positive	Positive	Negative	Positive	Positive	Positive
Starch Hydrolysis	Negative	Positive	Negative	Negative	Positive	Positive	Negative	Negative
Lipid Hydrolysis	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Table 3. List of identified bacterial strains

S.No.	Bacterial Isolate	Identified as
1.	*PSTB1*	*Enterobacter* sp.
2.	*PSTB2*	*Serratia* sp.
3.	*PSTB3*	*Enterobacte rium* sp.
4.	*PSTB4*	*Providencia* sp.
5.	*PSTB5*	*Raoutlella* sp.
6.	*PSTB6*	*Pectobacterium* sp.
7.	*PSTB7*	*Enterobacterium* sp.
8.	*PSTB8*	*Morganella* sp.

Table 4. Pectinolytic activity of isolated bacteria (Pectin Clear Zone) :

S. No	*Bacterial isolate*	*PCZ (mm)*
1	*Enterobacter* sp.	34
2	*Serratia* sp.	26
3	*Enterobacterium* sp.	22
4	*Providencia* sp.	15
5	*Raoutlella* sp.	18
6	*Pectobacterium* sp.	16
7	*Enterobacterium* sp.	18
8	*Morganell* sp.	18

Conclusion:

In the present study, the production of pectinase from *Enterobacter* sp. has been investigated under submerged fermentation. The high productivity of pectinase 82.647 U/ml, under suitable fermentation conditions suggested that *Enterobacter* sp. is a good pectinase producer than the earlier reported bacterial isolates. In addition to these properties, some additional properties like external carbon source (Fructose) and added vitamins (Riboflavin), low substrate concentration, less incubation time for pectinase production indicating the potential of *Enterobacter* sp. to be used at commercial level in fruit processing industries. This is the first ever report of pectinase production using mango industrial waste powder basal medium. An overview of the results obtained show that simple submerged fermentation of mango fruit processing industrial waste was suitable to produce low cost, high value product i.e., Pectinase by *Enterobacter* sp. The mango fruit processing industrial waste used as whole and sole carbon Source for the production of pectinase while the pectinase itself can find a number of applications in the mango processing industry.

Acknowledgments:

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References:

1. Chapter-IV. World scenario. Indian horticulture database 2011 for India data, March 2011.
2. Purnachandra Reddy, M. Saritha, K. V. (2013). Potential applications of 'King of Fruits' (Mango). Indo American journal of pharmaceutical research, 3(9).
3. Satvinder singh dhillon, Rajwant kaur gill, Sikander singh gill& Malkiat singh (2004). Studies on the utilization of citrus peel for pectinase production using fungus *Aspergillus niger.* International Journal of Environmental Studies, 61(2).
4. Berardini et al.,(2005). Utilization of mango peels as a source of pectin, polyphenolics. Innovation Food Science Emerging Technology, 6:442–452.
5. Mudgett, A.E., Demain, A.L. and Solomon, N.A. (1986). Manual of Industrial Microbiology and Biotechnology. American Society for Microbiology Washington,D.C., 66-83.
6. Smith and Aidoo, (1988). Growth of fungi on solid substrates. Physiology of industrial fungi. Blackwell, Oxford, England, 249-269.
7. Shahera H. Attyia and Sanaa M. Ashour (2002). Biodegradation of agro-industrial orange waste under solid stat fermentation and natural environmental conditions. Egyptian journal of biology,4: 23-30.

8. Ceci, L. and Loranzo. J. (1998). Determination of enzymatic activities of commercial pectinases for the clarification of apple juice. Food chemistry,61: 237-241.

9. Thakur B. R. (1997). Chemistry and uses of pectin. Crit. rev. food sci. nutrition, 37:47.

10. Aneja, K. R. (2010). Experiments in microbiology and plant pathology and biotechnology. (4th edition), New age international publishers, pp. 276-294.

11. Miller, Gail Lorenz (1959). Use of di-nitrosalicylic acid reagent for determination of reducing sugar. Annals of chemistry, 31 (3): 426-428.

12. Geetha, M., Saranraj, P., Mahalakshmi, S. and Reetha, D. (2012). Screening of pectinase producing bacteria and fungi for its pectinolytic activity using fruit wastes.International journal of biochemistry & biotech science, 1: 30-42.

13. Reda A. Bayoumi, Hesham M. Yassin, Mahmoud A. Swelim, Ebtsam Z. Abdel-All (2008). Production of bacterial pectinase(s) from agro-industrial wastes under solid state fermentation conditions. Journal of applied sciences research, 4(12): 1708-1721.

14. Ramachandran Sandhya and Kurup, G. (2013). Screening and isolation of pectinase from Fruit and vegetable wastes and the use of orange waste as a substrate for pectinase production. International research journal of biological sciences, 2(9): 34-39.

15. Bohdziewiez, J. and Bodzek, M. (1994). Ultrafiltration preparation of pectinolytic enzymes from citric acid fermentation broth. Proceedings of biochemistry, 29: 99-107.

16. Kashyap, D.R. Vohra, P.K. Chopra, S. and Tewari, R. (2001). Applications of pectinases in the commercial sector: a review. Bioresource technology, 77: 215-227.

17. Mehta, S.A., Rana Mitali, Shaikh Nilofer and Patel Nimisha (2013). Optimization of physiological parameters for pectinase production from soil isolates and its applications in fruit juice clarification. Journal of environmental research and development, 7 No. 4A.

18. Mukesh kumar, D J. Saranya, G M. Suresh, K. Andal Priyadharshini, D. Rajakumar, R. and Kalaichelvan, PT. (2012). Production and optimization of pectinase from *Bacillus* sp. MFW7 using cassava waste. Asian journal of plant science and research, 2 (3):369-375.

19. Sunnotel, O. and Nigam, P. (2002). Pectinolytic activity of bacteria isolated from soil and two fungal strains during submerged fermentation. World journal of microbiology & biotechnology, 18: 835–839.

20. Zeni, J. Cence, K. Grando, C. E. (2011). Screening of pectinase-producing microorganisms with polygalacturonase activity.Applied biochemistry biotechnology, 163 (3):383-92.

21. http://www.bio-world.com/productinfo/2_31_835_227/9086/Pectinase.html. “Cost of Pectinase-(10 g)”. 21560019-2- $117.33.

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Epidemiological survey of risk factors and prevalence of cervical cancer in Andhra Pradesh, India

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Abstract

To evaluate the epidemiological survey and the prevalence of cervical cancer in Andhra Pradesh, India. The incidence of cervical cancer data were extracted from Medical Colleges and its associated hospitals from 2009 to 2012. Amongst all the surveyed cases (6,971) 29.5% are cervical cancer cases with 28.1% of stage I, II and 71.8% cases are in advanced stages. Among the suspected cases, Pap smear results indicated that the 27.3% of cases are adenocarcinoma *in situ* and following atypical squamous cell of undifferentiated significance. Majority surveyed patients use tobacco products (20.1%) followed by contraceptive pills (17.95%) and areca nut products (6.2%). In conclusion, cervical cancer was the second most leading cancer among the women in Andhra Pradesh and its occurrence is mainly due to the infection of human papilloma virus and then followed by bacterial and fungal infections and other risk factors respectively.

Key Words: Areca nut, Cervical Cancer, Contraceptive Pills, Pap smear, Risk factors.

Introduction

Cancer is one of the leading causes of adult deaths across the worldwide especially developing counties like India (1). The exact number of cancer deaths in India is expected to increase because of increased population growth. Rates of cancer deaths are expected to rise, particularly from increases in the age-specific cancer risks of tobacco smoking, which increase the incidence of several types of cancer (1). India is a culturally and traditionally diverse country, with huge regional and rural-to-urban variation in lifestyles and in age-specific adult death rates. Thus, understanding the geographical and social distribution of specific cancers is essential to target cancer control programmes and spur further research into the causes of cancer. There are 24 urban population-based cancer registries in India, with only two registries representing rural areas (2). All the urban Population Based Cancer Registries such as Bangalore, Bhopal, Chennai, Delhi and Mumbai have shown a statistically significant decrease in prevalence of cancer (2). Since over 70% of the Indian population exist in the rural areas, cancer of uterine cervix still constitutes the number one cancer in females (3). The HPV virus takes very long progression time from mild dysplasia to carcinoma makes cervical cancer a relatively easily preventable disease and provides the rationale for screening. Most women who develop cervical cancer tend to have one or more recognizable co-factors that increase their risk efficiency for the disease. It is uncommon but not impossible for women to develop cervical cancer without any of these risk factors (4).

According to GLOBOCAN 2012, global burden rises to 14.1 million new cases and 8.2 million cancer deaths in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008. A significant increase was noticed in breast and

cervical cancers in 2012 (5,6). There will be almost 20 million new cases were estimated by the end of year 2020. It is not only in the number of new cases that will increase but also the proportion of new cases from the developing countries like India will also rise to around 70%. The magnitude of the problem of cancer in Indian Sub-Continent is disturbing (7). Though the cancer incidence rate in India is less than that of the Western countries but due to the large population size, number of cases is more prevalent at any time (8). The most common cancers among females are breast, cervix, ovary, oesophagus and mouth. Of this, cervical cancer is the second most common cancer among women worldwide after breast cancer. World Health Organization (WHO) reported that the cervical cancer comprises 12% of all cancers in women and it is the leading gynaecological malignancy in globally (9). It is an important public health problem especially for adult women in developing countries (10). The risk of cervical cancer remains high in many developing countries mostly due to the lack or inefficiency of existing prevention programmes.

Therefore, the aim of the present study was to evaluate the epidemiological survey of risk factors and prevalence of cervical cancer in Andhra Pradesh. The survey was based on the data available from medical colleges and its associated hospitals along with some cytopathological investigations in two medical colleges.

Methodology for Epidemiological survey

Medical Colleges and their associated Hospitals (Sri Venkateswara Medical College, Sri Venkateswara Meternity Hospital, Tirupati, Guntur Medical College Guntur, PES Medical College Kuppam) present in the Andhra Pradesh, India were involved in order to conduct the cervical cancer/Pre-cancer prevalence. Clinical-epidemiological survey is based on the questionnaire elucidated in table 1, 2 and 3. Tables also shows that the total number of patients was arranged according to the age pattern with characteristic features of type and stage of the tumor, cervical data includes the cervical smears pH, Whiff test and presence or absence of microbial infections. The present survey contains two components firstly, knowledge about types of cancers, cancer stage, cervical data (pH, Whiff test), secondly, risk factors of cervical cancer including microbial infection along with number of childrens, pre or post menopause (11).

Results

In the present investigation, Table-1 shows that all the cancer and cervical cancer cases were arranged according to the year and stage wise. Among all the surveyed cancer cases (6971), 29.5% cases are cervical cancer (2058) with 28.1% of stage I, II (579) and 71.8% cases are in advanced stages (stage III, IV) (1479).

Table-2 shows that the surveyed cervical cases were arranged according to the age, type of their cells, stage and cervical swab data. Among all the cervical cancer cases 35.9% cases are Squamous Cell Carcinoma (SCC) and 27.3, 22.4% are adenocarcinoma and Squamous Cell adenocarcinoma respectively with affect age maximum at 60-69 years and minimum affect at the age of 30-39 years. Based on the cancer stage, maximum cases are identified at the age of 60-69 years. In 92.8% cervical cases, vaginal pH was observed as greater than the 5, which indicates that the most of the cases shows increased pH due to the presence of anaerobic bacteria. Approximately 98.2% cases were positive for Ames test. Of the surveyed cervical cases, 38.6% cases were affect the bacterial infections and then followed by fungal (25.8%) and fungi with candida infection (26.1%) respectively.

Epidemiological studies have identified a number of risk factors such as infection with high risk human papillomaviruses (HR-HPV), contraceptive pills (12) and usage of tobacco, areca nut products and other factors. Majority of the cases were due to the HR-HPV, 17.95% patients were used contraceptive pills, 6.2%

patients were used tobacco products, 20.1% patients were used areca nut products (Table-3). Table-3 also shows that increased number of childrens also increases the prevalence of cervical cancer. In the present study, nearly 54% of cases are having more than 2 childrens and 9.4% of cervical cancer patients have no children.

Table-4 indicated the, patients with gynaecological problems and suspected cervical cancer cases (Abnormal cervical cytology with undifferentiated pap smear results) reported only from two medical colleges (Guntur Medical College, Guntur and PES Medical College, Kuppam). Among all the suspected cases (2841 cases) 27.2% of cases are adenocarcinoma *in situ* and followed by Atypical squamous cell of undifferentiated significance, low grade squamous intraepithelial lesions and high grade squamous intraepithelial lesions (22.6%, 18.6%, 18.0 %) respectively.

Discussion

India has a population of approximately 1.2 billion and accounts for a signiûcant burden of cervical cancer in the Indian subcontinent (3). There is an estimated annual global incidence of 5,00,000 cancers, in that India contributes 1,00,000 i.e., one-fifth of the world burden (13). A total of 4304 cervical cancer cases were registered during 1982-89 in the Chennai registry, India. In 1990, twenty percent of all female deaths from cancer in India, were from cervical cancer, amounting to an estimated 6,100 deaths (14). In 1996, cervical cancer accounted for 2,47,000 deaths in women. Approximately 20,000 new cases were detected in India, in the year 2000 (15). Recently a report says that there are an estimated 1.32 lakh new cases and 74 000 deaths annually in India (16). Particularly, in Southern India, carcinoma of the uterine cervix is the most common form of cancer in females (13).

The HR-HPV prevalence rates varied between 23-80%, but mostly above 20%. In the present study, the surveyed patients were at the age group of 30-79 years with a mean age of 56.8 years. 29.8% of the patients are under the middle age (55-65 years) with significant cancer characters, hence increased age increases the prevalence of the cancer, especially the median age at 58.5 shows the maximum occurrence of the disease.

In cytopathological studies of the cervical swab samples indicates that the 38.6 % of cases were infected with bacterial infections and 25.8% cases were infected with fungi and 26.1% infections were considered as mixed infections containing fungi with candida. Bacterial infection involves the lower genital tract and is characterized by the replacement of *Lactobacilli* predominant flora with *G. vaginalis*, anaerobes and *Mycoplasma hominis*.

Kian Behbakht and co-workers (17) reported that the elevated levels of microbial population especially bacterial infections in swab smears of cervical cancer patients. In the present study also 38.6 % cases were infected with bacterial infection, most of these bacteria creates an anaerobic environment which increases the normal pH of the vagina from 4 to above 8 (18). The anaerobic bacteria present in the vagina produce mucin degrading enzymes which are responsible for the entry of HPV virus and causes the precancerous stage. Another important finding stated that the nitrosamines produced by the bacteria play a major role in the development of pre-cancerous stage (19).

The present study showed that the most of the patients with bacterial infections were develops the precancerous stage of cervix with CIN properties. The similar types of results were also reported at Nam *et al.* (20) who showed that the incidence of CIN changes was significantly higher in women with bacterial infections.

The development of cervical cancer was also associated with the number of pregnancies or number of children's. HPV infected women who gave 2 or more births have twice the chance of developing cervical cancer compared to those who have no child (21). Higher level of knowledge was observed regarding the different risk factors

of cervical cancer by the usage of carcinogenic products. Of the surveyed patients 17.9% patients were used contraceptive pills to prevent the pregnancy, 20.1% patients were used areca nut products and 6.2% patients were used tobacco products. In concern to the Pap smear test and cytopathological studies similar findings were observed in study of Saha *et al.* (2010) (22) and Teresa Joy *et al.* (2011) (23). The products of tobacco and areca nut also produce nitrosamines which facilitate the increased rate of cervical cancer in the patients who frequently use the products of tobacco and areca nut.

Cervical cancer Trends in India and Andhra Pradesh State

The recent Indian censes (2011), shows that the population of India was the second largest in the world, with an estimated 1.2 billion people. Approximately 950,000 incidences of cancer cases and 6,34,000 associated mortalities reported in India in 2008 (24). Overall, the most commonly reported types of cancer in India were cervical, breast, lip/oral cavity, lung, and oesophagus (24). The maximum mortality rates, across both sexes were reported for cervical and breast cancer. According to India's recent population census, if India's population continues to grow at an annual rate of 1.4%, the population of India will likely more than that of China after 2030 years and leads to nearly one-fifth of the global population live in India, potentially making cervical cancer the single most common cancer in the world (25).

Across the India, rates of cervical cancer vary vastly by population, including those defined by geographic and demographic features. In India the second highest age specific rate of cervical cancer was reported among women in state of Andhra Pradesh, India (10). The information of cancer pattern and burden in India is based on the population based cancer registries covering approximately 4% of the total population. The estimated age standardized cervical cancer incidence and mortality rate around 30.7 and 17.8 per 100,000 respectively in 2002. The peak incidence was observed in the age of 70 years old women (26). Statistical data showed that the estimated number of cancer cases in Asian and Indian woman is about 28,51,110 and 948,858 respectively with 23.3% of death rate in Indian Women (27).

There is no National Cancer Registry Programme (NCRP) or other cancer registry in Andhra Pradesh state and neither Cancer Incidence in Five Continents (CI5) publishes the data for Andhra Pradesh state. The Cancer Atlas of India publishes the data about Andhra Pradesh, for only 2 out of 23 districts.

Cervical Cancer Screening in India

Cervical cancer is preventable when it was identified as early as possible, but most women in poorer countries do not have access to effective screening programmes. There are no organized screening programs in any province or region of India. Screening of asymptomatic women is practically absent in well-organized health care programs of the industrial and military sectors (28). In India, it has been estimated that even with a major effort to expand cytological examinations, it will not possible to screen even one-fourth of the population once in a lifetime in the near future (29).

Cytology is offered sporadically to women in selected urban areas attending health services for other reasons, but not as routine screening of asymptomatic women. In 2002, according to a World Health Organization (WHO) health survey, 2.6% of 4,586 women aged 18–69 years, ever had a Pap smear (30). It is estimated that less than 1.5 million smears are opportunistically taken annually.

Incidence and Mortality Patterns in India

A large body of evidences stated that the cancer patterns and burden in India is based on the projections from 18 population-based cancer registries covering approximately 4% of the population, including three rural registries in different regions. Age standardized cervical cancer incidence rates range from 9 to 40 per 1,00,000 women in various regions of India (31).

The estimated age-standardized cervical cancer incidence and mortality rates around 2002 were 30.7 and 17.8 per 10,00,00 women respectively. The highest incidence was observed in older women 70 years of age (26). The number of maternal deaths and cervical cancer cases is almost equal in India (30).

There is substantial awareness, advocacy and investment to reduce maternal deaths among policy makers, governments, professional societies (including the Federation of Obstetrics & Gynaecology Societies of India (FOGSI), social organizations and women's movements. It is paradoxical that there is very limited awareness on cervical cancer as a threat to the health of middle-aged women in the most productive period of their life.

Recommendations

In the present survey and investigations contributed towards the much needed lag in the knowledge of cervical cancer, there is an urgent need to diagnosed or screen at early stage of the cervical cancer. In order to increase the knowledge of risk factors of cervical cancer, there is need for cervical cancer screening awareness program in rural and urban areas. More health education about cervical cancer could be made an integral part of different levels of health care systems in Andhra Pradesh, India.

This may also help to increase the knowledge of risk factors as well as remove the misconceptions about the cervical cancer. The current survey highlights about cervical cancer, its screening and prevention amongst the patients. Implementation of the screening educational sessions was successful in improving their knowledge. Women especially at the age of 50-60 years, properly aware of this disease and can educate masses, increase the health seeking behaviour in women and thus reduce the burden of cervical cancer.

Conclusion

In conclusion cervical cancer was the second most leading cancer among all the cancers in Andhra Pradesh. Epidemiological survey also concluded that contraceptive pills, tobacco products are the main risk factors for the development of cervical cancer.

Conflict of Interest

The authors declare that there is no conflict of interests for this article.

Acknowledgements

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Table 1. Year wise data on all cancers along with cervical cancer during the period of 2009 to 2012: shows that 29.5% cases are cervical cancer with 71.8% cases are detected at advanced stages (Stage III & IV); 28.1% cases are diagnosed at stage I & II.

Year	Total Cancer cases	Cervical Cancer (%)	Stage –I &II (%)	Stage –III &IV (%)
2009	1098	494 (44.9)	103 (20.8)	391 (79.1)
2010	1910	562 (29.4)	168 (29.8)	394 (70.1)
2011	2114	546 (25.8)	193 (35.3)	353 (64.6)
2012	1849	456 (24.6)	115 (25.2)	341 (74.7)
Total	6971	2058 (29.5)	579 (28.1)	1479 (71.8)

Note: The data present in the table was extracted from surveyed Hospitals and their associated Medical Colleges. The data present within the parentheses is percent over total number of Patients.

Table 2. Characteristics of cervical cancer patients with different types, stages and microbial infections based on age factor include Squamous Cell Carcinoma; Adeno Carcinoma; cancer stage according to the TNM classification. Table also shows the pH and Whiff tests of the cervical swabs and microbial infections include bacterial fungal, fungi with candida.

S.No	Age(Years)	Type of Biopsy				Cancer Stage (TNM)		Cervical data		Microbial Infections				
		SCC	AC	SCC+AC		Others	I & II	III & IV	pH	Whiff tests		BI	FI	F+C	WI
								d" 5	> 5	P	N				
1	30-39	103	73	57	40	51	222	56	217	268	5	104	82	68	19
2	40-49	138	83	74	52	74	272	24	324	334	12	162	96	80	10
3	50-59	172	120	106	40	114	324	36	402	432	6	150	126	110	52
4	60-69	216	178	132	89	237	378	20	593	608	7	256	171	119	68
5	70-79	110	109	94	73	103	283	12	374	381	5	123	58	162	42
6	Total	739	563	463	294	579	1479	148	1910	2023	35	795	533	539	191
7	%	35.9	27.3	22.4	14.2	28.1	71.8	07.1	92.8	98.2	01.7	38.6	25.8	26.1	09.2

Note: SCC: Squamous Cell Carcinoma; **AC**: Adeno Carcinoma; **SCC+AC**: Squamous Cell Carcinoma+Adeno Carcinoma. **P**: Positive; **N**: negative; **BI**: Bacterial Infection; **FI**: Fungal Infection; **F+C**: Fungal with Candida Infection; **WI**: Without infection; **%**: Percent over Total number of Patients. The data present in the table extracted from surveyed Hospitals and associated Medical Colleges.

Table 3. Characteristics of cervical cancer patients with risk factors include that number of children, pre- and post-menopause; usage of contraceptive pills, tobacco, areca nut products and others.

S. No	Age	No.of Childrens			Menopause		Usage of Carcinogenic products			
		Nil	d" 2	> 2	Pre	post	C. Pills	Tobacco	Areca	others
1	30-39	54	93	126	273	-	68	37	74	94
2	40-49	16	115	217	323	23	56	15	94	183
3	50-59	35	154	249	32	406	84	26	85	243
4	60-69	47	263	305	12	603	101	32	72	408
5	70-79	43	129	214	-	386	61	18	90	217
6	Total	195	754	1111	640	1418	370	128	415	1145
7	%	09.4	36.6	53.9	31.0	68.9	17.9	06.2	20.1	55.6

Note: C.Pills: Contraceptive Pills; **%**: Percent over Total number of Patients (2058). The data present in the table was extracted from surveyed Hospitals and their associated Medical Colleges.

Table 4. Year wise data on Pap smear test results during 2009 to 2012 shows that 27.2% cases are Adeno Carcinoma *In Situ* followed by Low-grade (22.6%), high grade (18.6%) Squamous Intra epithelial Lesions; Atypical Squamous Cell Carcinoma (18.0%).

Year	Suspected cases					
	ASCUS (%)	**LSIL (%)**	**HSIL (%)**	**ACIS (%)**	**Negative (%)**	**Total**
2009	106 (14.8)	187 (26.1)	107 (14.9)	205 (28.6)	110 (15.3)	715
2010	127 (17.9)	163 (22.9)	105 (14.8)	216 (30.4)	98 (13.8)	709
2011	136 (18.8)	159 (22.0)	163 (22.6)	185 (25.6)	78 (10.8)	721
2012	145 (20.8)	134 (19.2)	154 (22.1)	167 (23.9)	96 (13.7)	696
Total	514 (18.0)	643 (22.6)	529 (18.6)	773 (27.2)	382 (13.4)	2841

Note: ASCUS: Atypical Squamous Cell Carcinoma; **LSIL**: Low-grade Squamous Intra epithelial Lesions; **HSIL**: High-grade Squamous Intra epithelial Lesions; **ACIS**: Adeno Carcinoma *In-Situ*. **Note:** The data present in the table was extracted from surveyed Hospitals and their associated Medical Colleges. The data present within the parentheses is percent over total number of Patients.

References

1. Jha, P. (2009). Avoidable global cancer deaths and total deaths from smoking. Nat Rev Cancer. 9: 655–64.
2. National Cancer Registry Programme (2010). Three-Year Report of Population Based Cancer Registries 2006–2008: Incidence and Distribution of Cancer. Bangalore: Indian Council of Medical Research.
3. Nandakumar, A., Ramnath, T. and Chaturvedi, M. (2009). The magnitude of cancer cervix in India. Indian J Med Res. 130: 219-221.
4. Ushadevi, G., Arul Anne R. and Ashok Kumar, T. (2012). Knowledge about Cervical Cancer Risk Factors among the Rural Women of Kancheepuram District, Tamil Nadu. Nat. J. Res. Com. Med. 1(2).
5. Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D. and Bray, F. Cancer Incidence and Mortality Worldwide: 2013; IARC Cancer Base No. 11.
6. Bray, F., Ren, J.S., Masuyer, E. and Ferlay, J. (2013). Global estimates of cancer prevalence for 27 sites in the adult population in 2008. Int. J. Cancer. 132(5):1133–1145.
7. Rao, D.N. and Ganesh, B. (1998). Estimate of cancer incidence in India in 1991. Indian J Cancer. 35: 10-8.
8. Krishnan Nair, M. and Sankaranarayanan, R. (1991). Epidemiological lead to cancer control. Cancer causes Control. 2: 263-265.
9. Senapathy, J.G., Umadevi, P. and Kannika, P.S. (2011).The present scenario of cervical cancer control and HPV epidemiology in India: an outline. Asian Pacific J. Cancer Prev. 12:1107-1115.
10. Parkin, D.M., Whelan, S.L., Ferlay, J., Teppo, L. and Thomas, D.B. (2002). Cancer incidence in five continents. IARC Scientific Publication. 8: 155.
11. Varen, A., Ozkilinc, G., Guler, A. and Oztop, I. (2008). Awareness of breast and cervical cancer risk factors and screening behaviours among nurses in rural region of Turkey. European Journal of Cancer Care. 17: 278–284.
12. Franceschi, S., Rajkuma,r T., Vaccarella, S., Gajalakshmi, V., Sharmila, A., Snijders, P.J., et al. (2003). Human papillomavirus and risk factors for cervical cancer in Chennai, India: A case-control study. Int J Cancer 107:127-33.
13. Shanta, V. (2003). Perspectives in Cervical Cancer prevention in India. The International Network for Cancer Treatment and Research.
14. Gajalakshmi, V., Rajaraman, S. and Shanta, V. (2000). A survival study of cancer in

Chennai, India. Indian J. Cancer. 37: 158-164.

15. Mandal, R., Mittal, S. and Basu, P. (2003). Management of cervical pre-cancer. In 'Detection, diagnosis and management of cervical intraepithelial neoplasia'. Eds Lodha ND, Kamal MM. Nagpur, 1.
16. Priyanka, G. (2009). A new vaccine for cervical cancer and some good hope. Available at:http://cancersupport. aarogya. com/index.php? option= com_content & task= view & id=333 & Itemid=361.
17. Kian, B., Jennifer, F., Ira, H., Alla, A., Jose, S. and Sebastian, F. (2002). Role of the vaginal microbiological ecosystem and cytokine profile in the promotion of cervical dysplasia: a case–control study. Infect. Dis. Obstet. Gynecol. 10:181–186.
18. Subramanyam, D., Raju Naidu, D.S., Wudayagiri, R. and Lokanatha, V. (2014). Effect of Concurrent Radio chemotherapy and Chemotherapy on Serum Proteins for Prospective Predictors of Patients with HPV Induced Cervical Cancer. Biomedicine & Pharmacotherapy. 68 : 315–320.
19. Subramanyam, D., Rajendra, W. and Lokanatha, V. (2014). Evaluation of microbial enzymes in normal and abnormal cervicovaginal fluids of cervical dysplasia: a case control study. Biomed Research International, Article ID 716346: 1-6.
20. Nam, K.H., Kim, Y.T., Kim, S.R., Kim, S.W., Kim, JW., Lee, M.K., Nam, E.J. and Jung, Y.W. (2009). Association between bacterial vaginosis and cervical intraepithelial neoplasia. J. Gynecol. Oncol. 20(1):39-43.
21. Munoz, N., Franceschi, S., Bosetti, C., Moreno, V., Herrero, R., Smith, J.S., Shah, K.V., Meijer, C.J. and Bosch, F.X. (2002). Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. Lancet. 359(9312):1093-1101.
22. Saha, A., Nag Chaudhury, A., Bhowmik, P. and Chatterjee, R. (2010). Awareness of Cervical Cancer Among Female Students of Premier Colleges in Kolkata, India. Asian Pacific Journal of Cancer Prevention. 11: 1085-1090.
23. Teresa, J., Brijesh, S., Chacchu, B. and Jenny, C. (2011). Awareness of Cervix Cancer Risk Factors in Educated Youth: A Cross-Sectional, Questionnaire Based Survey in India, Nepal, and Sri Lanka. Asian Pacific Journal of Cancer Prevention. 12: 1707-1711.
24. Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C. and Parkin, D. (2010). Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10. International Agency for Research on Cancer, Lyon, France. globocan.iarc.fr
25. Robin, M.B. (2012). Screening for cervical cancer: an exploratory study of urban women in Tamil Nadu, India. Ph.D Dissertation, University of Iowa.
26. Ferlay, J., Bray, F., Pisani, P. and Parkin, D.M. (2004). Cancer Incidence, Mortality and Prevalence Worldwide. IARC Cancer Base No. 5 Version 2.0. Lyon: IARC Press.
27. Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. and Parkin, D. (2010). Estimates of worldwide burden of cancer in 2008: Globocan 2008. Int. J. Cancer. 127: 2893–917.
28. Gheit, T., Vaccarella, S., Schmitt, M. et al., (2009). Prevalence of human papillo-mavirus types in cervical and oral cancers in central India. Vaccine. 27: 636-639.
29. Stjernsward, J., Eddy, D., Luthra, U.K. and Stanley, K. (1987). Plotting a new course for cervical cancer in developing countries. World Hlth. Frm. 8: 42-45.
30. World Health Organization Vaccine Preventable Diseases Monitoring System (2008). Available at: http://www.who.int/vaccines/global summery/immunization countrypro. leselect.cfm
31. Cancer Atlas India (2008). Available at: http://www.canceratlasindia. org/chapter6.asp.

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Evaluation of Antibacterial activity of Seed Extracts of *Sesamum indicum*

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Abstract

The present research work was focused on the antibacterial activity of seed extracts of *Sesamum indicum*. The aqueous and ethanolic extracts of seeds prepared from various extraction procedures were evaluated against a Gram positive bacteria *Bacillus subtilis* and a Gram negative bacterial strain, *Escherichia coli* by agar well diffusion method. Preliminary phytochemical screenings of the extracts were also done. Various concentrations (100 µg/ml, 200 µg/ml and 300 µg/ml) of the extracts were incorporated into the agar wells. A single dose of standard tetracycline (300 µg/ml) was also placed in the well. Both the extracts showed concentration dependant activity against the microbes. The results were analyzed based upon the readings of diameter of zones of inhibition. Both the seed extracts showed better inhibitory activity against gram negative bacteria. The test substances were compared with that of the standard drug tetracycline. The ethanolic extract showed significant activity against the test organisms than that of the aqueous extract. It was concluded that the seed extracts of *Sesamum indicum* showed better anti bacterial activity and further studies were suggested to isolate the active principles that were responsible for the activity.

Key words

Antibacterial, *Sesamum indicum*, Zone of inhibition, *Bacillus subtilis*, *Escherichia coli*, Tetracycline

Introduction

Antibacterials are the agents which are used for the treatment of infections caused by various bacterial species. The frequency of life threatening infections caused by the pathogenic microbes has increased all over the world (1). The increasing prevalence of multi-resistant strains of bacteria and the strains with reduced susceptibility to bacteria evoked the search for new infection fighting strategies (2). Plants are the sources of many potent and powerful drugs (3). Many studies were done worldwide to investigate the antibacterial property of plants as they are the cheapest and safer alternatives (4, 5, 6). Considering such potentiality of plants, a systematic investigation was undertaken to screen the locally available plant *Sesamum indicum* for the antibacterial activity.

Sesamum indicum, belonging to the family pedaliaceae, is an annual branching plant with 4 – 5 feet height. The shape of the leaves varies. The flowers were reddish white, single and were located in the axils of the leaves. The fruit is an oblong capsule with small oval blackish seeds. Sesame oil is used in the preparation of iodinol and brominol which were employed for the external, internal and subcutaneous use. It is also used in making some liniments, plasters and ointments. They are used well in the treatment of different cases like infantile diarrhoea, dysentery, helminth and microbial infections. The oil is said to be a laxative and is also used to promote menstruation (7). Hence the present

study was undertaken specifically to investigate the role of *Sesamum indicum* seed extracts as antibacterials.

Materials and Methods

Plant material

***Collection and authentication of plant materials*:** The seeds of *Sesamum indicum* belonging to the family Pedaliaceae were collected in the month of August, 2013 from the local areas of Guntur district, Andhra Pradesh, India. The plant material was identified and authenticated by Dr. Sreenivasa prasanna, M. Pharm., PhD, Department of Pharmacognosy, M. L. College of Pharmacy, Singarayakonda, Andhra Pradesh, India and a voucher specimen (MPESFPS – 02/13) has been deposited.

***Processing of sample*:** The seeds of *Sesamum indicum* were collected, dried at ambient conditions and pulverized into fine powder which is used for the extraction process. Here maceration and soxhelation processes were employed for the extraction of seeds.

Preparation of extracts

***Preparation of aqueous extract of Sesamum indicum seeds*:** The powdered dried seeds were subjected to extraction process by maceration with distilled water at room temperature for 48 hours with occasional stirring. The extract was filtered after 48 hours, then concentrated to dryness at room temperature and preserved.

***Preparation of ethanolic extract of Sesamum indicum seeds*:** The powdered dried seeds were loaded into the soxhlet extractor and subjected to extraction with ethanol. After extraction, the solvent was distilled off, concentrated to dryness at room temperature and preserved.

***Phytochemical analysis*:** The aqueous and ethanolic extracts of *Sesamum indicum* seeds were subjected to preliminary phytochemical screening by using various phytochemical tests for qualitative analysis of presence of various constituents like carbohydrates, fixed oils, glycosides, alkaloids, flavonoids, tannins, polyphenols, steroids and saponins (8, 9). The various tests include

Test for Carbohydrates

a. ***Molisch's test:*** Aqueous or alcoholic solution of the extract was added with 10% aqueous solution of alpha naphthol and shakened. Concentrated sulphuric acid was added along the sides of the tube. Violet ring at the junction of two liquids shows the presence of carbohydrates.

b. ***Benedict's test:*** 5 ml of benedict's reagent was added to 3 ml of test solution, boiled for 2 minutes and allowed to cool. Greenish yellow or red precipitate shows presence of reducing sugars.

c. ***Barfoed's test:*** 2 ml of test solution was added to 2 ml of barfoed's reagent and boiled. Brick red precipitate shows the presence of monosaccharides.

Test for fixed oils: The presence of fixed oils was determined by placing 5 ml of test solution in an ordinary paper. Observation of a translucent spot indicates the presence of fixed oils.

***Test for Glycosides*:** A pinch of the extract was treated with glacial acetic acid and few drops of ferric chloride solution, followed by the addition of concentrated sulphuric acid. Formation of a red ring at the junction of two liquids indicates the presence of glycosides.

***Test for alkaloids*:** To the extracts, dilute hydrochloric acid was added and filtered. The filtrate was treated with the extracts.

a. ***Mayer's test:*** When the filtrate was treated with mayer's reagent, potassium mercuric iodine solution, appearance of cream coloured precipitate indicated the presence of alkaloids.

b. ***Hager's test:*** The filtrate when treated with hager's reagent, picric acid, appearance of yellow coloured precipitate indicated the presence of alkaloids.

Test for flavonoids

a. ***Zinc hydrochloride test:*** To the extract, a mixture of zinc dust and concentrated hydrochloric acid was added. Appearance of red colour after few minutes indicates the presence of flavonoids.

b. ***Shinoda's test:*** The extract was dissolved in alcohol and to that a few magnesium turnings were added followed by concentrated hydrochloric acid drop wise and heated. Appearance of magenta colour shows the presence of flavonoids.

Test for tannins and polyphenolic compounds

a. ***Ferric chloride test:*** 5% W/V solution of ferric chloride in 90% alcohol was mixed up with the test solution. Appearance of violet colour indicates the presence of phenols.

b. ***Lead acetate test:*** 10% lead acetate solution was mixed up with the test solution. Appearance of white precipitate indicates the presence of tannins.

Test for steroids

a. ***Liebermann – Burchard's test:*** The extract was treated with 3 ml of acetic anhydride, few drops of glacial acetic acid followed by a drop of concentrated sulphuric acid. Appearance of bluish green colour indicates the presence of steroids.

b. ***Salkowski's test:*** The extract was treated with 3 ml of acetic anhydride, few drops of concentrated sulphuric acid. Appearance of yellow colour indicates the presence of steroids.

Test for saponins

Foam test: 1 ml of extract was diluted to 20 ml with distilled water and shaken well in a test tube. The formation of foam in the upper part of the test tube indicates the presence of saponins.

***Test microorganisms*:** The bacterial strains which were used in the present study are Gram-positive, namely, *Bacillus subtilis* and a Gram-negative, namely, *Escherichia coli*. The strains were obtained from the Department of Biotechnology, Bapatla College of pharmacy, Bapatla, Andhrapradesh, India.

***Screening of Antibacterial activity*:** The agar well diffusion method was used to determine the inhibitory effects of the *Sesamum indicum* seed extracts against the microbes (10). The bacterial isolates obtained were first grown in a nutrient broth for 18 hrs at 37^0C. Simultaneously, nutrient agar medium was prepared and sterilized. Then it was poured into sterile petri dishes while hot and then allowed to set. After ensuring that the medium was solidified, 0.2 ml of broth culture of the bacteria was aseptically inoculated on this plate using a sterile cotton swab and allowed to dry (11). A uniform growth was ensured for accurate results. Wells of 6 mm size was made in the agar plates with the help of the sterile cork borer and these wells were loaded with different concentrations (100 µg/ml, 200 µg/ml, 300 µg/ml) of the aqueous and ethanolic extracts of *Sesamum indicum* seeds. A standard (tetracycline) solution which acts as a positive control was also placed (300 µg/ml) in an extra well in each of the dishes. Methanol was used for the dilution of the extracts as well as the standard drug. Then these plates were incubated at 38^0C for 24 to 48 h. After the incubation time, the zones of inhibition were measured in millimeter diameter using a meter rule (12).

Minimum Inhibitory Concentration (MIC): The lowest concentration of the plant extract required for inhibiting the growth of the bacterial strain was considered as the minimum inhibitory concentration (MIC). The minimum inhibitory concentration for both the gram-positive and gram-negative organisms was found by agar streak dilution method (13). Stock solutions of aqueous and ethanolic extracts of *Sesamum indicum* were mixed with the known quantity of molten sterile agar media aseptically. About 20 ml of the media containing the extract was poured into each sterile petri dish and allowed to solidify. Microorganisms were then streaked one by one on the agar plate aseptically. After streaking, all

the plates were incubated at 37 ± 1^{0}C for 24 hours and the plates were observed for the growth of the microorganism. The lowest concentration of the plant extract at which the MIC was observed and noted.

Results and Discussion

***Physical properties of the extracts*:** The aqueous extract was prepared using maceration, whereas the ethanolic extract was prepared using soxhelation. The aqueous and ethanolic extracts of *Sesamum indicum* showed better yields. The aqueous extract which was blackish, showed a yield of 19.62% with semisolid texture. Whereas the ethanolic extract which was brownish black, showed a yield of 15.5% with oily texture. As the soxhlet apparatus is favourable for volatile solvents, water is not used here for the purpose of extraction. Hence, the aqueous extract was obtained from maceration process, but not by soxhelation. The ethanolic extract was dried completely at 80^{0}C until a constant weight was obtained. As the boiling point of ethanol is 78.3^{0}C, and here the extract was boiled at a temperature more than 78^{0}C, there wouldn't be any alcoholic residue in the extract. It confirms that the residual ethanol doesn't interfere in the evaluation of antibacterial activity.

***Phytochemical analysis*:** Preliminary phytochemical screening of aqueous and ethanolic extracts of *Sesamum indicum* was performed by using various phytochemical identification tests. These tests revealed the presence of carbohydrates, alkaloids, glycosides, saponins, flavonoids, polyphenols and tannins in both the aqueous and ethanolic extracts of *Sesamum indicum*. Whereas the ethanolic extract showed the presence of fixed oils and steroids in addition. The results of preliminary phytochemical screening of aqueous and ethanolic extracts of *Sesamum indicum* were tabulated in table – 1.

***Antibacterial activity*:** The zone of inhibition of the seed extracts were compared with that of the zone of inhibition of standard drug. Both the aqueous and ethanolic extracts of *Sesamum indicum* showed better action against the Gram-negative bacteria (*Escherichia coli*) than that of the Gram-positive organisms (*Bacillus subtilis*). The standard drug tetracycline showed a zone of inhibition of 20 mm in case of Gram-positive organisms and 24 mm in case of Gram-negative organisms. As the doses of the extracts increased, an increase in the antibacterial activity was observed which was indicated by the increase in the zone of inhibitions of both the extracts. At a maximum dose of 300 µg/ml, the ethanolic extract of *Sesamum indicum* showed clear zone of inhibitions of 16 mm and 22 mm against the gram-positive and gram-negative organisms respectively. Whereas at the same dose, the aqueous extract of *Sesamum indicum* showed zone of inhibitions of 14 mm and 20 mm against the gram-positive and gram-negative organisms respectively. This clearly indicates that the ethanolic extract of *Sesamum indicum* showed better antibacterial activity than that of the aqueous extract. These results indicating the antibacterial activity of aqueous and ethanolic extracts of seeds of *Sesamum indicum* against *Bacillus subtilis* and *Escherichia coli* were

Table 1. Phytochemical screening of aqueous and ethanolic extracts of seeds of *Sesamum indicum*

Phytochemical Constituents	*Sesamum indicum*	
	Aqueous Extract	Ethanolic Extract
Carbohydrates	+	+
Fixed oils	–	+
Glycosides	+	+
Alkaloids	+	+
Flavonoids	+	+
Tannins	+	+
Polyphenols	+	+
Steroids	–	+
Saponins	+	+

+ indicates presence; – indicates absence of the phytochemical constituents which were screened using various identification tests.

Table 2. Antibacterial activity of aqueous and ethanolic extracts of seeds of *Sesamum indicum* against *Bacillus subtilis* and *Escherichia coli*

Bacteria	Concentration of the plant extracts (µg/ml)	Zone of Inhibition (in mm)		Positive control Tetracycline (300 µg/ml) (in mm)
		Aqueous extract	Ethanolic extract	
Bacillus subtilis (Gram positive)	100	8	10	20
	200	10	12	
	300	14	16	
Escherichia coli (Gram negative)	100	12	16	24
	200	16	20	
	300	20	22	

tabulated in table – 2. These results were thoughtful because in the present employed traditional method of treating a bacterial infection, preparing a plant extract with an organic solvent showed better antibacterial activity (14). In the whole study it was observed that the seed extracts showed high activity against a Gram-negative organism than that of a Gram-positive organism. The seeds of *Sesamum indicum* which were used for the study may contain the bio-components whose antibacterial potentials are highly comparable with that of the tetracycline against all Gram-negative and Gram-positive bacteria tested. The activity of the plant parts against both the Gram-negative and Gram-positive bacteria may be an indicative of the presence of broad spectrum antibiotic compounds in the plant (15). Many studies have supported this statement (16, 17, 18). The use of plants to heal diseases, including infectious one, has been extensively applied by the people (19). Today most of the pathogenic organisms are becoming resistant to antibiotics (20). To overcome this alarming problem, the discovery of novel active compounds against new targets is a matter of urgency. Thus *Sesamum indicum* could become a promising natural antibacterial agent with potential applications in pharmaceutical industry for controlling the pathogenic bacteria.

***Minimum inhibitory concentration*:** The minimum inhibitory concentration values of both aqueous and ethanolic extracts were noted. The lowest MIC values were observed for aqueous extract at 88 – 91 µg/ml and for ethanolic extract at 92 – 95 µg/ml against the bacteria tested.

Conclusion

From the present study, it was concluded that both the aqueous and ethanolic extracts of seeds of *Sesamum indicum* possesses potent antibacterial activity which supports the traditional folklore. Further work will emphasize the isolation and characterization of active principles responsible for the antibacterial activity in clear and to establish the effectiveness and pharmacological rationale for the use of *Sesamum indicum* as an antibacterial agent.

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References

1. Albari, M.A., Syeed, M.A., Rahman, M.S. and Mossadik, M.A. (2006). Characterization and antimicrobial activities of a phenolic acid derivative produced by *Streptomyces bangladeshiensis*, a novel species collected in Bangladesh. Research Journal of Medicine and Medical Sciences, 1: 77 – 81.

2. Mashiar, R.M., Mominul, I.S.M., Shamima, A.S., Soriful, I.M., Atikur, R.M. and Mizanur, R.M. (2009). Antibacterial Activity of Leaf Juice and Extracts of *Moringa oleifera* Lam. against Some Human Pathogenic Bacteria. CMU Journal of Natural Sciences, 8(2): 219 – 227.

3. Mahesh, B. and Satish, S. (2008). Antimicrobial activity of some important medicinal plant against plant and human pathogens. World Journal of Agricultural Sciences, 4(S): 839 – 843.

4. Adriana, B., Almodovar, A.N.M., Pereira, C.T. and Mariangela, T.A. (2007). Antimicrobial efficacy of *Curcuma zedoaria* extract as assessed by linear regression compared with commercial mouth rinses. Brazilian Journal of Microbiology, 38: 440 – 445.

5. Doughari, J.M., El-mahmood, A.M. and Manzara, S. (2007). Studies on the antibacterial activity of root extracts of *Carica papaya* L. African Journal of Microbiology and Research, 37 – 41.

6. Pretorius, C.J. and Watt, E. (2001). Purification and identification of active components of *Carpobrotus edulis* L. Journal of Ethnopharmacology, 76: 87 – 91.

7. Warrier, K., Nambiyar, P.K. and Ramankutty, C. (2006). Indian Medicinal Plants: A compendium of 500 species, Orient Longman Private Limited, Chennai, India, pp. 54.

8. Trease, G.E. and Evans, W.C. (1983). Textbook of Pharmacognosy, Balliere & Tindall, (12th edition), London, UK, pp. 348 – 383.

9. Khandelwal, K.R. (2003). Practical Pharmacognosy, Nirali Prakashan, Pune, India, pp. 149.

10. Ntiejumokwu, S. and Aiemika, T.E. (1991). Antimicrobial and phytochemical investigation of the stem bark of *Boswellia dalzielli*. West African Journal of pharmacology, Drug Research, 10: 100 – 104.

11. Marimuthu, M., Uma, S. and Gurumoorthi, P. (2013). Comparative phytochemical evaluation and antimicrobial activity of two different germplasm of *Mucuna*. International Journal of Pharmacy and Pharmaceutical Sciences, 5(2): 46 – 51.

12. Adebayo, M.F., Akinpelu, D.A. and Okoli, A.I. (2008). The bioactive and phytochemical properties of g. Kola (heckel) seed extract on some pathogens. African Journal of Biotechnology, 7(21): 3938 – 3948.

13. Stella, R., Madhusudhan R. G., and Anjana D. I. (2013). Evaluation of Antimicrobial activity of leaves of Gisekia pharnaceoides. International Journal of Pharmacy and Pharmaceutical Sciences, 5(2): 540 – 542.

14. Nair, R., Kalariya, T. and Sumitra, C. (2005). Antibacterial activity of some selected Indian medicinal flora. Turkish Journal of Biology, 29: 41 – 47.

15. Siddhuraju, P. and Becker, K. (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agro-climatic origins of drumstick tree (*Moringa oleifera* Lam.). Journal of Agriculture and Food Chemicals, 15: 2144 – 2155.

16. Sandeep, D. (2014). Evaluation of antibacterial activity of seed extracts of Vigna unguiculata. International Journal of Pharmacy and Pharmaceutical Sciences, 6(1): 75 – 77.

17. Tim, C.T.P. and Andrew, J.L. (2005). Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents, 26: 343 – 356.

18. Pavithra, V.K. and Bhagya, L.O. (2010). Antibacterial activity of black pepper (*Piper nigrum* Linn.) with special reference to its mode of action on bacteria. Indian Journal of Natural Products and Resources, 1(2): 213 – 215.

19. Gislene, G.F.N., Juliana, L., Paulo, C.F. and Giuliana, L.S. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Brazilian Journal of Microbiology, 31(4): 15 – 19.

20. Chandarana, H., Baluja, S. and Chanda, S.V. (2005). Comparison of antibacterial activities of selected species of zingiberaceae family and some synthetic compounds. Turkish Journal of Biology, 29: 83 – 97.

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Microwave mutagenesis for altered lactic acid production in *Lactobacillus plantarum,* and *Streptococcus mutans*

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Abstract

This study attempted at microwave mutagenesis of: (i) *Lactobacillus plantarum* for lactic acid overproduction, and (ii) *Streptococcus mutans* for reduced lactic acid production. Lactic acid is among the microbiological products with high market potential. Lactic acid is also an important virulence factor in formation of dental caries by *S. mutans*, as the acid produced by the bacteria leads to demineralisation of the teeth. Two of the mutants obtained (one from each organism) were able to maintain the altered lactic acid production till 10 generations. However the magnitude of alteration in lactic acid producing ability of the mutants went on decreasing over generations. The microwave effects observed in this study seem largely to be athermal in nature. Investigation of the mutants obtained at molecular level may result in identification of novel mutations responsible for altered lactic acid production. These mutations can then be introduced into a suitable organism either for better industrial production of lactic acid, or for constructing new probiotic strain(s) for possible application in maintenance of oral health.

Keywords: Microwave mutagenesis, Lactic acid, Probiotic, Microwave specific athermal effect**.**

Introduction

The part of electromagnetic spectrum corresponding to wavelength range of 300 MHz to 300 GHz is referred as microwave (MW) region. During last few decades this non-ionizing part of electromagnetic spectrum has experienced widespread uses (1-2), e.g. in telecommunications (3), domestic and medical microwave devices for diagnosis and/or therapy (4, 5), extraction (6, 7), sterilization (8-10), waste treatment (11), etc. MW have been described to exert two kinds of effects on biological systems, i.e. thermal and athermal (MW specific non-thermal effect). The former has been established and understood very well, whereas controversy has built up surrounding the latter (12). More research is required on the athermal MW effects, whether they affect biological entities, their possible mode of action, and if these effects are heritable (13).

One of the recently indicated applications of MW is their use as a mutagenic agent. Lin *et al.* (14) reported MW induced overproduction of lactic acid in *Lactobacillus rhamnosus*. Kothari *et al*. (15) has reported mutagenic effect of MW radiation on exopolysacchride production in *Xanthomonas campestris*. Li *et al*. (16) reported enhanced cellulase production in *Trichoderma viride* mutated by compound mutagenesis using MW (2450 MHz; 700 W for 15-195 s) and ultraviolet. Radiation mutagenesis on account of its convenience, safety, and better mutagenicity results is an attractive alternative to conventional strain improvement strategies based on use of mutagenic chemicals,

transposons or viruses. At times industrial strains can exhibit intolerance to ultraviolet and X-ray radiation. MW mutagenesis can emerge as a clean and effective tool for strain improvement. It can also help get rid of the problem of photo-reactivation, often observed with ultraviolet mutagenesis (17).

The present study attempted at MW mutagenesis of: (i) *Lactobacillus plantarum* for lactic acid overproduction, and (ii) *Streptococcus mutans* for reduced lactic acid production. Lactic acid is among the microbiological products with high market potential. Lactic acid has found application in foods, beverages, biodegradable polymer production, and hence bioproduction of L- lactic acid has become an issue of significance (18). The production of biodegradable plastic polylactide has led to increased interest in optically pure lactic acid, accounting for recent shift from chemical to microbial processes. Though lactic acid has been on the market for sometime, further strain improvement for the large scale microbial production processes is needed (19).

Lactic acid is an important virulence factor in formation of dental caries by *S. mutans*, as the acid produced by the bacteria leads to demineralisation of the teeth (20). The mutant and/or recombinant strains capable of producing lower or no lactic acid can find application as a probiotic in maintenance of oral health. Probiotic methods are currently under focus as an alternative means of caries management (21).

Materials and Methods

Test Organisms: *L. plantarum* (MTCC 2621), and *S. mutans* (MTCC 497) were procured from Microbial Type Culture Collection (MTCC), Chandigarh.

Microwave Treatment: Bacterial suspension was prepared in sterile normal saline, from an active culture growing on brain heart infusion agar (BHI; HiMedia, Mumbai) (22, 23), and MRS agar (HiMedia) (24, 25, 14), in case of *S. mutans* and *L. Plantarum* respectively. Inoculum turbidity was adjusted to that of 0.5 McFarland standard. Test culture (5 mL) in sterile screw capped glass vials (15 mL, Merck) was exposed to MW radiation (90 W/ 270 W/ 450 W; 2450 MHz) in a domestic MW apparatus (Electrolux® EM30EC90SS). MW treatment at 90 W was given for three different time durations viz. 2, 4, and 6 min; MW treatment at 270 W was given for 2, and 5 min; whereas MW treatment at 450 W was given for 2 min. Vials inside the MW apparatus were placed in an ice containing beaker, so as to avoid/minimize any thermal heating. Temperature of the microbial suspension after MW treatment at 90 W did not go beyond 15°C; while using MW at 450 W (2 min), temperature reached 50.10 ± 0.41°C; at 270 W (2 min) temperature reached 29.50 ± 0.50°C, and at 270 W (5 min) it was 67.80 ± 0.62°C. The whole MW treatment was performed in an air-conditioned room. Untreated inoculum was used as control. Before MW treatment all the inoculum vials (including control) were put in ice for 5 min to nullify any variations in initial temperature. Test organism was immediately (in less than 5 min) inoculated onto BHI or MRS agar for *S. mutans* and *L. plantarum* respectively, following MW treatment. Incubation was made at 35°C under static condition for 48 h.

Lactic acid estimation: Lactic acid was estimated using the photometric method described by Kimberley and Taylor (26) with some modification (27). Briefly, after measuring the growth at 625 nm, culture broth was centrifuged (Nüve NF 800 R) at 7500 rpm for 15 min, and the resulting supernatant was used for lactic acid estimation. One mL of this supernatant (after appropriate dilution) was mixed with 1 mL of 20% $CuSO_4$ (HiMedia) solution and 8 mL water, followed by addition of 1 g of calcium hydroxide (CDH, Delhi). This mixture was incubated at room temperature for 30 min, followed by centrifugation at 7500 rpm for 15 min. One mL of the resulting supernatant was mixed with 0.05 mL of 4% $CuSO_4$ solution, followed by addition of 6 mL concentrated H_2SO_4 (Merck, Mumbai). After proper mixing the reaction mixture was placed

in a boiling water bath for 5 min, followed by cooling and addition of 0.1 mL of *p*-hydroxy diphenyl reagent [1.5% *p*-hydroxy diphenyl (HiMedia) in 95% ethanol]. It was allowed to stand at room temperature for 30 min, and then in a boiling water bath for exactly 90 seconds. After cooling OD was measured at 560 nm, and lactic acid concentration was calculated from a standard curve obtained using pure lactic acid (HiMedia) at 10-30 μg/mL.

Screening for mutants : Following the MW treatment of *L. plantarum* suspension, the treated inoculum was streaked on MRS agar plate, and incubated at 35°C for 48 h. After the incubation 3 colonies from each plate corresponding to different MW treatments were picked randomly, and each colony (a separate code was given to each picked colony) was streaked on to a separate MRS agar plate. Daughter populations thus generated from a single parent colony were then inoculated into the MRS broth, followed by incubation at 35°C for 48 h under static condition. After incubation, lactic acid estimation was made for all the MW treated inoculums. Then the plates corresponding to the MW treatment yielding higher lactic acid were selected for further experiments. Subculturing was done from the plates of overproducing mutant(s), and daughter population resulting from each subculturing was checked for its lactic acid production (in comparison to the wild type), upto 10 generations. Similar screening was done with *S. mutans* for reduced lactic acid production, using BHI as the growth medium.

Statistical analysis: All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD). Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. *P* values less than 0.05 were considered to be statistically significant.

Results and Discussion

Lactobacillus plantarum: *L. plantarum* was given three different MW treatments viz. 270 W for 2 min, 270 W for 5 min, and 450 W for 2 min. When the MW treated inoculum corresponding to 270 W (5 min) was plated on MRS agar plate, no growth appeared after incubation, suggesting that due to high temperature cells were killed during MW treatment. From the plates corresponding to remaining two MW treatments, three colonies (designated as A, B, and C) were picked from each plate, and streaked on to a new MRS agar plate (one plate from each colony). Inoculum made from the resulting growth was inoculated into MRS broth, followed by lactic acid estimation after incubation. Four of the six selected colonies were found to produce significantly higher lactic acid compared to the wild type control (Table 1). Interestingly, despite considerable change in the lactic acid producing ability of the MW treated cultures, no notable change occurred with respect to growth and pH (except a minor reduction in pH for one of the colonies picked from 270 W treatment). This indicates that in the lactic acid overproducing cultures, per cell lactic acid production and/or excretion was much higher than the wild type control.

From these four overproducing colonies, we selected the three (viz. the colonies at serial number 3, 5, and 6 in Table 1) showing highest magnitude of lactic acid overproduction. These overproducing colonies were designated as VHVS_LP, VHVS_LP2, and VHVS_LP1 respectively. Lactic acid production in these three colonies (i.e. in their daughter populations) was studied up to 10 generations, to investigate whether they retain the trait of lactic acid overproduction, or revert back to parent phenotype. Results of this study over multiple generations are presented in Table 2.

Among the three selected colonies, at the selection stage VHVS_LP2 registered maximum lactic acid overproduction (15.47 times higher than wild type) followed by VHVS_LP1 (9.47 times higher than wild type) and VHVS_LP (9.15 times higher than wild type). VHVS_LP2 could maintain significant lactic acid overproduction only till 2nd generation, and experiments with it

were discontinued after 3rd generation. VHVS_LP1 could maintain lactic acid overproduction till 7th generation. VHVS_LP was able to maintain lactic acid overproduction till 10th generation, however the magnitude of overproduction kept on shrinking over generations (Fig 1). Though at the 10th generation VHVS_LP showed much lesser overproduction (6.66%) as compared to the overproduction at the selection stage (815.78%), this relatively smaller overproduction was still statistically significant (p<0.05). This mutant was confirmed for its identity by subjecting it to 16 s rRNA sequencing, and this sequence was submitted to GenBank with accession no. KJ690777 (http://www.ncbi.nlm.nih.gov/nuccore/KJ690777.1).

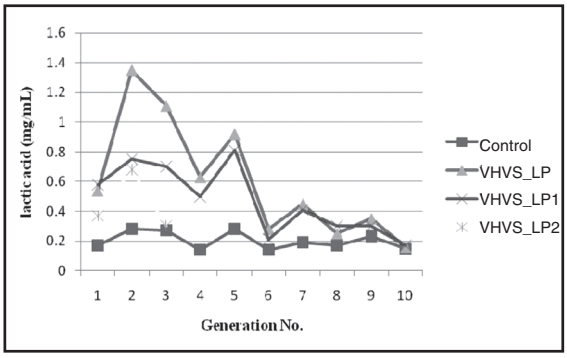

Fig. 1. Lactic acid production over multiple generations of overproducing mutants of *L. plantarum*
Control: wild type strain (with no MW exposure)
VHVS_LP, VHVS_LP1, and VHVS_LP2 are the overproducing mutants obtained after MW exposure.

Throughout these experiments the mutants showed considerable variation with respect to lactic acid production in comparison to the wild type, but there were no such major variations with respect to pH and growth, in general. This indicates that the observed lactic acid production resulted from increased production and/or secretion of lactic acid in the mutant cells, without growth potential of the mutants getting much affected. One of the bottlenecks in the way of industrial lactic acid production is the physiological demand of keeping the pH relatively higher (between 5 and 7) (19). It may be possible that the mutants obtained in this study were perhaps capable of continuing lactic acid production for some more time, even after the pH went below 5. Additionally, the mutants might be producing lower amount of by-products such as succinic acid, making diversion of more sugar flux possible into lactic acid synthesis.

Table 1. Effect of MW exposure on pH, growth and lactic acid production in *L. plantarum*

No.	MW treatment (Power and time duration)	Colony code	pH (Mean±SD)	% change compared to control	Growth (OD_{625}) (Mean±SD)	% change compared to control	Lactic acid (mg/mL) (Mean± SD)	% change compared to control
1.	0 min (control)	-	4.50±0.01	0.00	0.55±0.001	0.00	0.19±0.00	0.00
2	270 W (2 min)	(A)	4.47±0.07	-0.66	0.55±0.002	0.00	0.79±0.28	315.78
3		(B)	4.45±0.07	-1.11*	0.55±0.002	0.00	1.74±0.26	815.78*
4		(C)	4.46±0.03	-0.88	0.55±0.002	0.00	0.75±0.09	294.73*
5	450 W (2 min)	(A)	4.47±0.02	-0.66	0.55±0.001	0.00	1.80±0.16	847.36**
6		(B)	4.52±0.00	0.44	0.55±0.001	0.00	2.94±0.00	1447.36**
7		(C)	4.53±0.03	0.66	0.55±0.000	0.00	0.17±0.00	-10.52**

*p<0.05; **p<0.01; minus sign indicates a decrease over control

Table 2. Lactic acid production over multiple generations of selected overproducing mutants of *L. plantarum*

Colony designation	pH (Mean ± SD)	% Change compared to control	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Lactic acid (mg/mL) (Mean ±SD)	% Change compared to control
			1st Generation			
Control	4.26±0.007	0.00	0.819±0.062	0.00	0.17±0.01	0.00
VHVS_LP (270W/2 min; A)	4.23±0.00	-0.70	0.709±0.018	-3.55	0.54±0.03	217.64**
VHVS_LP2 (450W/2 min; A)	4.08±0.00	-4.22	0.700±0.0141	-14.53*	0.37±0.09	117.64
VHVS_LP1 (450W/2 min; B)	4.27±0.00	0.23	0.575±0.0381	-29.80*	0.58±0.03	241.17**
			2nd Generation			
Control	4.27±0.00	0.00	1.59±0.09		0.28±0.04	0.00
VHVS_LP	4.24±0.00	-0.70*	1.47±0.01	-8.55	1.35±0.09	382.14**
VHVS_LP2	4.22±0.00	-1.17	1.65±0.01	3.77	0.68±0.01	142.85**
VHVS_LP1	4.29±0.00	0.46*	1.52±0.00	-4.41	0.75±0.12	167.85*
			3rd Generation			
Control	4.21±0.04	0.00	1.54±0.00	0.00	0.27±0.00	0.00
VHVS_LP	4.14±0.00	-1.66	1.46±0.007	-4.88**	1.11 ±0.26	311.11*
VHVS_LP2	4.13±0.00	-1.90	1.21±0.0141	-21.43**	0.30±0.14	11.11
VHVS_LP1	4.14±0.02	-1.66	1.51±0.0424	-1.95	0.70±0.21	159.25
			4th Generation			
Control	4.47±0.00	0.00	1.76±0.000	0.00	0.14±0.03	0.00
VHVS_LP	4.39±0.01	-1.78*	1.75±0.007	-0.56	0.63±0.05	350.00**
VHVS_LP1	4.44±0.00	-0.67*	1.70±0.007	-3.12**	0.50±0.07	257.14*
			5th Generation			
Control	4.14±0.00	0.00	1.40±0.00	0.00	0.28±0.02	0.00
VHVS_LP	4.11±0.00	-0.72*	1.57±0.014	12.14**	0.92±0.03	228.57*
VHVS_LP1	4.15±0.00	0.24	1.50±0.028	7.14*	0.81±0.12	189.28*
			6th generation			
Control	4.18±0.01	0.00	1.66±0.00	0.00	0.14±0.03	0.00
VHVS_LP	4.18±0.02	0.00	1.68±0.02	1.20	0.28±0.01	100.00*
VHVS_LP1	4.18±0.01	0.00	1.66±0.25	0.00	0.21±0.01	50.00
			7th generation			
Control	3.96±0.00	0.00	1.42±0.000	0.00	0.19±0.01	0.00
VHVS_LP	3.91±0.00	-1.26*	1.43±0.014	0.70	0.45±0.03	136.84*
VHVS_LP1	3.90±0.00	-1.51	1.36±0.007	-3.87	0.41±0.05	115.78*
			8th generation			
Control	4.13±0.01	0.00	1.58±0.02	0.00	0.17±0.00	0.00
VHVS_LP	4.06±0.01	-1.69*	1.56±0.00	-1.26	0.25±0.07	47.05
VHVS_LP1	4.06±0.01	-1.69*	1.53±0.042	-3.16	0.30±0.14	76.47
			9th generation			
Control	4.11±0.00	0.00	1.65±0.014	0.00	0.23±0.07	0.00
VHVS_LP	4.03±0.00	-1.94**	1.65±0.014	0.00	0.35±0.07	52.17*
VHVS_LP1	4.10±0.00	-0.24	1.60±0.00	-3.03*	0.30±0.14	30.43
			10th generation			
Control	4.22±0.00	0.00	1.6±0.00	0.00	0.15±0.04	0.00
VHVS_LP	4.20±0.01	-0.47	1.64±0.00	2.50*	0.16±0.01	6.66*
VHVS_LP1	4.22±0.00	0.00	1.60±0.00	0.00	0.17±0.03	13.33

*$p<0.05$; **$p<0.01$; minus sign indicates a decrease over control; VHVS_LP2 could maintain significant lactic acid overproduction only till 2nd generation, and hence experiments with it were discontinued after 3rd generation.

Table 3. Effect of low power MW (90 W) on pH, growth and lactic acid production of *S. mutans*

MW treatment (min)	Colony code	pH (Mean ± SD)	% change compared to control	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Lactic acid (mg/mL) (Mean ± SD)	% change compared to control
0 (Control)	-	4.45±0.04	0.00	0.30±0.00	0.00	0.62±0.02	0.00
2	(A)	4.43±0.02	-0.44	0.35±0.04	15.21	0.48±0.00	-22.58*
	(B)	4.41±0.01	-0.89	0.34±0.00	10.35**	0.31±0.05	-50.00**
	(C)	4.41±0.01	-0.89	0.33±0.02	9.38	0.33±0.00	-46.77*
4	(A)	4.41±0.01	-0.89	0.33±0.04	7.76	0.16±0.04	-74.19**
	(B)	4.42±0.06	-0.67	0.34±0.02	11.32	0.17±0.03	-72.58**
	(C)	4.45±0.04	0.00	0.29±0.01	-6.14	0.34±0.05	-45.16*
6	(A)	4.44±0.02	-0.22	0.25±0.01	-18.77*	0.41±0.03	-66.12**
	(B)	4.42±0.01	-0.67	0.29±0.01	-3.23	0.23±0.00	-62.90**
	(C)	4.63±0.03	4.04**	0.19±0.00	-38.51**	0.20±0.00	-67.74**

*p<0.05; **p<0.01; minus sign indicates a decrease over control

Streptococcus mutans: *S. mutans* was exposed to MW at 90 W for three different time durations viz. 2 min, 4 min and, 6 min. Following the MW treatment, two BHI agar plates were streaked from each of the MW treated vials. From the plates corresponding to each MW treatment three colonies (designated as A, B, and C) were picked, and streaked on to a new BHI agar plate. Inoculum made from the resulting growth was inoculated into BHI broth, followed by lactic acid estimation after incubation. Out of these randomly selected 18 colonies, nine were found to produce significantly lower lactic acid compared to the wild type control (Table 3; data shown only for the colonies producing lesser lactic acid). For a *S. mutans* strain to be a good probiotic candidate, in addition to reduced lactic acid production it should also be having better growth potential than the wild type strain. The criteria of reduced lactic acid production was fulfilled by all the 9 isolates listed in Table 3, but only the isolate designated as (2B) was capable of growing better (10.35% higher growth) than the wild type, and hence it was selected for further experiments. Additionally one more isolate (2A) was also selected for further study; though not statistically significant, it registered the highest percentage increase in growth. Better growth or increased colonisation potential can make any particular strain better suited for probiotic applications (22), as upon use as a probiotic they are expected to compete with the pre-existing natural flora.

The two selected *S. mutans* isolates showing lesser lactic acid production were designated as VHVS_SM and VHVS_SM1, and their lactic acid production was investigated over multiple generations, whether they retain the trait of reduced lactic acid production. VHVS_SM retained its capacity to produce lesser lactic acid than wild type till 10th generation (Fig 2), along with a higher growth potential (Table 4). VHVS_SM1 could retain the trait of lesser lactic acid production only till 2nd generation, and hence experiments with this isolate were discontinued after third generation. Though the selected mutants had different growth and lactic acid production pattern than wild type, no major changes were noted with respect to pH of the culture broth at the time of harvest.

In this study two mutants have been obtained using MW mutagenesis. First one (VHVS_LP) was a superior producer of lactic acid, and another one (VHVS_SM) was inferior

Table 4. Lactic acid production over multiple generations of selected underproducing mutants (VHVS_SM, and VHVS_SM1) of *S. mutans*

Colony designation	pH (Mean ± SD)	% Change compared to control	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Lactic acid (mg/mL) (Mean ±SD)	% Change compared to control
			1st Generation			
Control	4.50±0.03	0.00	0.42±0.07	0.00	0.16±0.01	0.00
VHVS_SM (2 min; A)	4.51±0.01	0.22	0.33±0.05	-21.43	0.09±0.02	-43.75*
VHVS_SM1 (2 min; B)	4.52±0.02	0.44	0.44±0.09	4.76	0.13±0.03	-18.75*
			2nd generation			
Control	4.33±0.09	0.00	0.10±0.00	0.00	0.27±0.01	0.00
VHVS_SM	4.36±0.00	0.69	0.21±0.00	108.86**	0.17±0.00	-37.03**
VHVS_SM1	4.38±0.01	1.15	0.20±0.01	90.65*	0.15±0.03	-44.44*
			3rd generation			
Control	5.53±0.00	0.00	0.67±0.00	0.00	0.24±0.01	0.00
VHVS_SM	5.57±0.01	0.72	0.70±0.00	3.53*	0.13±0.01	-45.83*
VHVS_SM1	5.55±0.00	0.36	0.71±0.00	4.86*	0.24±0.04	00.00
			4th generation			
Control	4.57±0.01	0.00	0.67±0.01	0.00	0.24±0.01	0.00
VHVS_SM	4.63±0.01	1.31*	0.70±0.01	4.74	0.13±0.02	-45.83*
			5th generation			
Control	5.61±0.00	0.00	0.83±0.12	0.00	0.24±0.04	0.00
VHVS_SM	5.64±0.03	0.53	0.70±0.01	-15.55	0.16±0.02	-33.33
			6th generation			
Control	5.37±0.02	0.00	0.53±0.01	0.00	0.27±0.01	0.00
VHVS_SM	5.39±0.09	0.37	0.67±0.03	26.72*	0.19±0.01	-29.62*
			7th generation			
Control	4.49±0.08	0.00	0.39±0.01	0.00	0.24±0.01	0.00
VHVS_SM	4.64±0.05	3.34	0.56±0.00	44.05**	0.18±0.01	-25.0*
			8th generation			
Control	4.55±0.01	0.00	0.43±0.02	0.00	0.26±0.02	0.00
VHVS_SM	4.55±0.01	0.00	0.53±0.01	23.48**	0.18±0.00	-30.76**
			9th generation			
Control	4.79±0.00	0.00	0.58±0.00	0.00	0.25±0.01	0.00
VHVS_SM	4.80±0.01	0.2	0.64±0.01	10.99**	0.18±0.01	-28.0*
			10th generation			
Control	4.55±0.00	0.00	0.68±0.00	0.00	0.28±0.00	0.00
VHVS_SM	4.57±0.02	0.43	0.71±0.01	4.41*	0.24±0.01	-14.28*

*p<0.05, **p<0.01; minus sign indicates a decrease over control
VHVS_SM1 could retain the trait of lesser lactic acid production only till 2nd generation, and hence experiments with this isolate were discontinued after third generation.

producer of lactic acid. These two were mutants of wild type strains of *L. plantarum* and *S. mutans* respectively. In both cases the magnitude of alteration in lactic acid production of the mutants was higher at the selection stage, and this magnitude underwent a reduction over generations, i.e. the mutation got partially (but not completely) reverted towards the parent phenotype. Other mutants (VHVS_LP1, VHVS_LP2, and VHVS_SM1) obtained reverted back to the parent phenotype much faster. Literature contains reports indicating reversible nature of MW effects, as well as, those suggesting the MW induced mutations to be stable. Kothari *et al*. (15) reported mutagenic effect of MW radiation on exopolysaccharide production in *Xanthomonas campestris,* however the xanthan overproducing mutants were shown to revert back to the parent phenotype. Pasiuga *et al*. (28) reported disappearance of low-level MW induced effects after few generations in *Drosophila melanogaster.* MW treatment might have a profound effect on mutation repair system of a cell for initial few generations, but thereafter the repair system may restore its efficiency. Exploitation of MW mutagenesis resulting in genetically stable mutants has also been reported by few workers. Lactic acid overproducing mutants of *Lactobaciilus rhamnosus* using MW radiations (2450 MHz; 400 W for 3 min) were obtained by Lin *et al*. (14), and these mutants were found to be stable for up to 9 generations. Li *et al.* (29) claimed *Kleibsella pneumoniae* mutants with superior nitrogen fixing and P-solubilising ability, obtained through MW (250 W for 36 s) mutagenesis, to be genetically stable.

Altered lactic acid production observed in the mutants reported in this study may be due to the effect of MW on lactic acid producing and/or secreting machinery of the cell. Bollet *et al*. (30) has reported alteration in the cell membrane permeability on account of MW treatment. Lactate is transported across the cell membrane of *S. mutans* as lactic acid in an electroneutral process independent of metabolic energy, and has important bioenergetic implications for the cell (31). As lactic acid production is important for the *S. mutans* cells from a bioenergetic point of view, even partial loss of the ability to produce lactic acid may not be favourable for them, and the mutants may tend to restore the lactic acid producing capacity typical of the wild type strain.

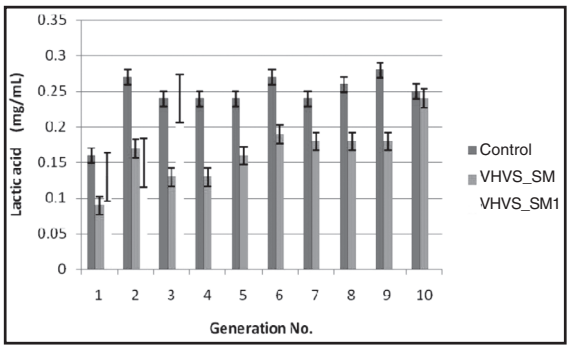

Fig. 2. Lactic acid production over multiple generations of the underproducing mutants of *S. mutans*

Control: wild type strain (with no MW exposure)
VHVS_SM and VHVS_SM1 are the underproducing mutant strains obtained after MW exposure.

Among the possible targets on which MW might have acted giving rise to mutation(s) are genes coding for lactate dehydrogenase, *ldh* open reading frame (ORF), etc. It is possible that MW treatment might have introduced multiple mutations in the genome of the mutant(s) obtained, all of these mutations may not be directly responsible for the observed modification in the lactic acid producing ability of our mutants. While comparing the lactic acid overproducing *L. rhamnosus* strains mutated by MW irradiation with the parent strains through AFLP analysis, Lin *et al*. (14) identified 51 bands of genomic DNA from mutated strains which were distinct from the parent strain, and predicted 45 genes with possible mutations. Among these 45 genes, three (malate/lactate dehydrogenase, pyruvate kinase, and NAD-dependent aldehyde dehydrogenase) were related to L-lactic acid production. Mutations in these and other such proteins involved in pyruvate metabolism can affect lactic acid production.

Acidogenicity has been proposed as the major virulence trait of the odontopathogenic bacterium *S. mutans* (32). Under conditions of sugar excess lactic acid is the major end-product of glycolysis by this bacterium. *S. mutans* strains with partial loss of lactic acid production may be able to still maintain somewhat acidic environment in the oral cavity restricting growth of other pathogens, but they may not produce so much of lactic acid which may lead to demineralization of the teeth enamel.

Conclusion

This study shows that MW radiation can be used as an effective mutagenic agent. During the MW treatments from which VHVS_LP and VHVS_SM were obtained, temperature did not go too high (not beyond 30ºC). Thus it can be said that the mutations observed were largely owing to the MW-specific athermal effect. These mutants should be subjected to further investigation including transcriptome and proteome analysis, so that mutations responsible for altered lactic acid production can be identified. Mutation(s) thus identified as responsible for lactic acid overproduction may then be introduced in industrial strains of lactic acid bacteria, paving the way for economically more favourable production of lactic acid. On the other line, mutation(s) identified as responsible for reduced lactic acid production can help in construction of useful effector strain(s) for use in replacement therapy of dental caries.

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References

1. Porcelli, M., Cacciapuoti, G., Fusco S., Massa, R., d'Ambrosio, G., Bertoldo, C., Rosa, M. and Zappia, V. (1997). Non-thermal effects of microwaves on proteins: thermophilic enzymes as model system. FEBS Letters 402: 102-106.
2. Mishra, T., Kushwah, P. and Kothari, V. (2013). Effect of low power microwave on bacterial growth, protein synthesis, and intracellular enzyme (glucose-6-phosphatase and β-galactosidase) activity. Biochemistry and Molecular Biology. 1(2): 27-33. DOI: 10.12966/bmb.08.01.2013
3. Belyaev, I. Y. (2005). Non-thermal biological effects of microwaves. Mikrotalasna Revija, 11(2): 13-29.
4. Hornback, N. B., Shupe, R. E., Shidnia, H., Joe, B. T., Sayoc, E. and Marshall, C. (1977). Preliminary clinical results of combined 433 megahertz microwave therapy and radiation therapy on patients with advanced cancer. Cancer, 40: 2854-2863.
5. Saito, K. and Ito, K. (2010). Preliminary study of coagulation monitoring by antenna for treatment during microwave coagulation therapy. The Open Biomedical Engineering Journal. 4: 13-15.
6. Mandal, V., Mohan, Y. and Hemalatha, S. (2007). Microwave assisted extraction—an innovative and promising extraction tool for medicinal plant research. Pharmacognosy Reviews, 1(1): 7-18.
7. Gupta, A., Naraniwal, M. and Kothari, V. (2012). Modern extraction methods for preparation of bioactive plant extracts. International Journal of Applied and Natural Sciences, 1(1): 8-26.
8. Sasaki, K., Honda, W., Shimizu, K., Lizima, K., Ehara, T., Okuzawa, K. and Miyake, Y. (1995). Microwave continuous sterilization of injection ampoules. PDA Journal of Pharmaceutical Science and Technology, 50(3): 172-179.
9. Bhattacharjee, M. K., Sugawara, K. and Ayandeji O. T. (2009). Microwave sterilization of growth medium alleviates inhibition of *Aggregatibacter actinomycetemcomitans* by Maillard reaction products. Journal of Microbiological Methods, 78(2): 227-230.
10. Kothari, V., Patadia, M. and Trivedi, N. (2011). Microwave sterilized media

supports better microbial growth than autoclaved media. Research in Biotechnology, 2(5): 63-75.

11. Lin, L., Yuan, S., Chen, J., Xu, Z. and Lu, X. (2009). Removal of ammonia nitrogen in wastewater by microwave radiation. Journal of Hazardous Materials, 161: 1063–1068.

12. Trivedi, N., Patadia, M. and Kothari, V. (2011). Biological applications of microwaves. International Journal of Life Sciences and Technology, 4(6): 37-46.

13. Kushwah, P., Mishra, T. and Kothari, V. (2013). Effect of microwave radiation on growth, enzyme activity (Amylase and Pectinase), and/or exopolysaccharide production in *Bacillus subtilis*, *Streptococcus mutans*, *Xanthomonas campestris* and *Pectobacterium carotovora.* British Microbiology Research Journal. 3(4): 645-653.

14. Lin, H., Chen, X., Yu, L., Xu, W., Wang, P., Zhang, X., ... and Ren, N. (2012). Screening of *Lactobacillus rhamnosus* strains mutated by microwave irradiation for increased lactic acid production. African Journal of Microbiology Research, 6(31): 6055-6065.

15. Kothari, V., Mishra, T. and Kushwah, P. (2014). Mutagenic effect of microwave radiation on exopolysaccharide production in *Xanthomonas campestris.* Current Trends in Biotechnology and Pharmacy, 8 (1): 29-37.

16. Li, X., Yang, H., Roy, B., Park, E., Jiang J., Wang D. and Miao Y. (2010). Enhanced cellulase production of the *Trichoderma viride* mutated by microwave and ultraviolet. Microbiological Research, 165(3): 190-198.

17. Zhang, J., Huang, L., Pei, Y. and Tian, L. (2005). Effects of mutation induced by microwave irradiation on antifungal activity of marine strain B1. Chinese Journal of Marine Drugs, 24; 1-5.

18. Kricheldorf, H. (2001). Synthesis of application of polylactides. Chemosphere, 43(1): 49-54.

19. Sauer, M., Porro, D., Mattanovich, D. and Branduardi, P. (2008). Microbial production of organic acids: expanding the markets. Trends in Biotechnology, 26(2): 100-108.

20. Argimo´n, S. and Caufield, W. (2011). Distribution of putative virulence genes in *Streptococcus mutans* strains does not correlate with caries experience. Journal of Clinical Microbiology, 49 (3): 984–992.

21. Anderson, M. (2006). A probiotic approach to caries management. Pediatric Dentistry, 28(2): 151-153.

22. Hillman, J., Brooks, T., Michalek, S., Harmon, C., Snoep, L. and Weijden, C. (2000). Construction and characterization of an effector strain of *Streptococcus mutans* for replacement therapy of dental caries. Infection and Immunity, 68(2): 543-549.

23. Kamiya, R., Napimoga, M., Hofling, J. and Goncalves, R. (2005). Frequency of four different mutacin genes in *Streptococcus mutans* genotypes isolated from caries-free and caries-active individuals. Journal of Medical Microbiology, 54; 599–604.

24. Altaf, M., Venkateshwar, M., Srijana, M. and Reddy, G. (2006). An economic approach for L-(+) lactic acid fermentation by *Lactobacillus amylophilus* GV6 using inexpensive carbon and nitrogen sources. Journal of Applied Microbiology, 103; 372-380.

25. Chauhan K., Trivedi U. and Patel K. (2007). Statistical screening of medium components by Plackett–Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice. Bioresource Technology, 98: 98–103.

26. Kimberely, A. C. and Taylor, C. (1995). A simple colorimetric assay for muramic acid and lactic acid. Applied Biochemistry and Biotechnology, 56: 49-58.

27. Jayaraman, J. (2011). Glycolysis. In: Laboratory Manual in Biochemistry (2nd edition), New age publishers, New Delhi, India, pp.153.

28. Pasiuga, V. N., Grabina, V. A., Bykov, V. N. and Shkorbatov, Y. G. (2007). Long-term effects of low-level microwave radiation on mutation frequency in *Drosophila*. In: Microwave & Telecommunication Technology, 2007. CriMiCo 2007. 17th International Crimean Conference,783-784.

29. Li, J., Zhang, S., Shi, S. and Huo, P. (2011). Mutational approach for N2-fixing and P-solubilizing mutant strains of *Klebsiella pneumoniae* RSN19 by microwave mutagenesis. World Journal of Microbiology and Biotechnology, 27(6): 1481-1489.

30. Bollet, C., Gevaudan, M.J., De Lamballerie, X., Zandotti, C. and De Micco, P. (1991). A simple method for isolation of chromosomal DNA from gram positive or acid-fast bacteria. Nucleic Acids Research, 19(8): 1955.

31. Dashper, S. G. and Reynolds, E. C. (1996). Lactic acid excretion by *Streptococcus mutans.* Microbiology, 142: 33-39.

32. Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. Microbiological Reviews, 50(4): 353-380.

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Proline Accumulates in Transgenic Red Banana (*Musa acuminate*Colla) Transformed with *P5CSF129A* Gene

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Abstract

Agrobacterium tumefaciens mediated genetic transformation in red banana (*Musa acuminata*Colla) is reported in the present communication.Male flower buds and suckers of red banana were transformed using *A. tumefaciens* strain LBA 4404 with the binary vector pCAMBIA1301, having hygromycin resistance gene as a selectable marker,*â-glucuronidase* (*GUS*) as a reporter and a mutated version of the gene *"*[1]*-pyrroline 5-carboxylate synthetase* (*P5CSF129A*) encoding a proline biosynthetic pathway enzyme P5CS (EC 2.7.2.11/ 1.2.1.41) isolated from *Vignaaconitifolia*. Male flower buds proved to be better explants than suckers for the regeneration and genetic transformation. Green shoots were recovered from *A. tumefaciens*-transformed explants on selection medium consisting of Murashige and Skoog's (MS) basal medium supplemented with 5 mg/L 6-benzylaminopurine (BAP), 2 mg/L indole-3-butyric acid (IBA), 250 mg/L cefotaxime and 25 mg/L hygromycin. Shoots were rooted on MS basal medium fortified with 3 mg/L IBA, 250 mg/L cefotaxime and 25 mg/L hygromycin. Transient expression of the GUS was observed in transgenic plants. The integration of the *P5CSF129A* gene was confirmed by polymerase chain reaction (PCR) and Southern blotting techniques. Transformation frequency was 11.3% and transgenic T_0 plants accumulated 2 to 3-folds higher proline when compared to untransformed control plants. This work possibly paves the way for exploiting other genes for engineering salt, drought and temperature stress tolerance in red banana.

Key Words: *Musa acuminata*, *P5CSF129A*, proline accumulation, genetic transformation

Introduction

Irrigated lands are particularly prone to salinization and salinity has profound effects on crop production (1). Increasing the salt tolerance of crop plants is vital for sustained food production. The response of plants to osmotic stress is complex, several physiological and biochemical changes take place and arrays of genes are induced. Several of these genes and their products have been identified; some are involved in the biosynthesis of osmolytes such as proline. Plants accumulate proline under osmotic stress (2, 3). The role of this amino acid during abiotic stress tolerance in plants has been widely discussed (4). Also, transgenic plants that accumulate high levels of proline are reported to display increased tolerance to salt and osmotic stress, as well as to cold (3, 5, 6, and 7). Various stress protection mechanisms have been proposed for proline. It is suggested to function as an osmolyte and take part in osmotic adjustment (3), as a stabilizer of sub-cellular structures, in protecting macro molecules (8), and as a free-radical scavenger (9). The synthesis of proline is initiated from glutamate which is dehydrogenated to glutamyl ã-semialdehide and spontaneously converted to Ä[1]-pyrroline-5-

carboxylic acid. Both steps are catalyzed by a bifunctional enzyme pyrroline-5-carboxylate synthetase (P5CS) which also behaves as a limiting step in proline biosynthesis (5). Moth bean (*Vignaaconitifolia*) cDNA clone encoding P5CS enzyme was first isolated by a functional complementation technique using *E. coli* mutants (10). Transgenic plants over-expressing the *P5CS* gene from *Vignaaconitifolia* accumulate high proline levels and are more tolerant to osmotic stress (3, 11, 12).

Banana (*Musa* sp.) is developing world's fourth most important field crop after rice, wheat and maize, in terms of gross production. The crop is grown in many countries throughout the tropics and sub-tropics, with an annual world production of around 95.6 million tons on an average area of 4.84 million hectares. India accounts for 27.4% of world's banana production with a yield of 26.2 million tons during 2010-11(13). It is grown in a range of environments and production systems and provides nutritionally staple food and a significant source of revenue all around the year. In India, bananas are grown in the states of Andhra Pradesh, Karnataka, Kerala, Maharashtra, Tamil Nadu and to some extent in western and central India. However, the development of new banana varieties through conventional breeding programs remains difficult because of sterility and polyploidy of most edible cultivars. Genetic transformation offers a viable means for introduction of agronomically important genes into popular high yielding lines. Therefore, an efficient regeneration and transformation protocol is crucial in selected varieties of banana for its improvement, particularly for drought and salt tolerance. Genetic transformation using *A. tumefaciens* is reported earlier (14, 15, 16, 17) and also by particle bombardment (18, 19). These reports include studies on transient expression of transformed genes, stable transformation and regeneration of transgenics and heritability of transgenes in banana. Since there are no reports of transformation in red banana thus far, present study aims at standardizing parameters for genetic transformation using *Agrobacterium*.

Materials and Methods

***Plant material*:** The terminal male flower buds and suckers were collected from the field grown plants and sterilized with 70% (v/v) absolute alcohol under sterile laminar airflow for one minute. The outer enveloping bracts were removed and inner parts (2-3 cm in length) containing male flower primordia were isolated and used as explants. These explants were used for genetic transformation after sterilizing with 0.1% $HgCl_2$ (w/v) for 5 minutes and washing with sterile distilled water 3-4 times to remove traces of $HgCl_2$.

***Agrobacterium strain, plasmid vector, culture, infection and co-cultivation* :** Transformation was performed using *A. tumefaciens*strain LBA4404 harboring the binary vector pCAMBIA1301. This construct contains *hptII*gene for resistance to hygromycinsulphate, *GUS* reporter gene encoding â-glucuronidase (GUS) intron and the mutated version of *Vignaaconitifolia P5CSF129A* cDNA under the control of CaMV35S promoter. To prepare the inoculum, a single colony of *Agrobacterium* was grown in 50 mL of liquid YEB medium containing 50 mg/L kanamycin for 24 h in dark at 28 ^{0}C, on a gyratory shaker (Orbiteck, India) at 200 rpm. Bacteria were then pelleted by centrifugation at 4,000 rpm for 5 min followed by resuspension in Murashige and Skoog's (MS) liquid medium[20] supplemented with 100 mM acetosyringone and the pH was adjusted to 5.7. The culture was allowed to grow till it attained an optical density (OD) of 0.5 at A_{600nm}($5x10^9$ cells/mL). One hour prior to co-cultivation of explants, 50-100 µM acetosyringone was added. A total of 300 explants were used for transformation. Explants were gently pricked few times to make wounds using a sterile needle. Excised male flower buds were immersed for 30 min in *Agrobacterium*. Explants were blotted dry using sterile Whatman Number 1 filter paper and inoculated (one explants/culture tube) onto regeneration medium consisting of MS basal salts supplemented with BAP (5 mg/L) and IBA (2 mg/L). Co-cultivation was performed for 0-5 days with an interval of 1 day, under a 16 h photoperiod

with a light intensity of 40 µmol m^{-2} s^{-1} and kept at 25 ± 2 ^{0}C.

Selection of transformants **:** Different concentrations (0-25 mg/L) of hygromycin were used in the present study to determine the lethal level at which wild type explants do not exhibit any growth. On medium supplemented with 25 mg/L, necrosis and death of explants was noticed, therefore, this concentration of hygromycin was used for selection of putative transgenics. After co-cultivation, the male flower buds were rinsed thoroughly with sterilized distilled water containing filter sterilized cefotaxime (250 mg/L), dried on sterile Whatman Number one filter paper and transferred to MS medium supplemented with 5 mg/L BAP + 2 mg/L IBA + 250 mg/L cefotaxime and 25 mg/L hygromycin. The explants with emerging shoot buds were subcultured on the same medium with the same composition for 4 weeks. Regenerated shoots (4-5 cm long) were transferred to MS medium supplemented with 3 mg/L IBA for root induction. Finally, the plantlets were transferred to plastic pots containing garden soil mixed with vermiculite and sand (2:1:1) for hardening and then subsequently transferred to green house.

Histochemical GUS staining, polymerase chain reaction (PCR) and Southern blot analysis **:** All putative transformants (T_0) were analyzed for GUSexpression following the method described by Jefferson (21). The transformed shoot tissues were immersed in 1 mM X-Gluc solution (Sigma-Aldrich, USA), 100 mM NaH_2PO_4 (pH 7.0), 0.5 mM potassium ferricyanide, and 0.1% triton X-100 and incubated in the dark overnight at 37 ^{0}C. After staining, the materials were treated with 70% ethanol for three days to remove chlorophyll before observation. Blue spots were recorded using a stereoscopic microscope. Genomic DNA from leaves of control and T_0 progeny of five transformants was extracted according to Doyle and Doyle (22). PCR reaction was performed with purified genomic DNA (30 ng) using two convergent primers that are complementary to the *P5CSF129A* DNA.

F: ACC ATA TGT GCT CTA AAG GCT ATT GC
R: GCG TCG ACG AAT TCC CGA TCT ATG AA

Amplification reactions consisted of one cycle of 4 min at 94 °C, followed by 25 cycles (60s, 94 °C, 45s 58 °C, 2 min 72 °C) and finally an extension cycle of 10 min at 72 °C[23]. The final volume of PCR reaction was 25 µl (F primer 0.5 µl, R primer 0.5 µl, dNTP 1 µl, Taq polymerase 1 µl, PCR buffer 5 µl, DNA sample 5 µl and distilled water 12 µl). PCR products were separated on 1% agarose gel and stained with ethidium bromide. Integration of the *P5CSF129A* gene in transgenic banana plants (T_0) was confirmed by Southern blot analysis. Twenty µg of genomic DNA was isolated from leaves of each PCR-positive transgenic plants along with untransformed controls and was digested with *Eco*RI. The samples were resolved on 0.8% agarose gel and blotted on a Hybond N+ nylon membrane (Bioscience, Little Chalfont, Bucks, UK) membrane, following the method described by Sambrook and Russell (23).

Proline measurement in T_0 plants**:** Proline content was determined according to the method of Bates et al. (24). One gram leaf tissue from transgenics (T_0) and untransformed control plants was quickly frozen and ground in liquid nitrogen, the tissue was extracted with 3% sulfosalicylic (m/v) coloring reaction containing ninhidrin solution and glacial acetic acid. The mixture was boiled until the colour developed. Colour was extracted with toluene, and the chromophore was measured with a spectrophotometer at ë 520.

Results and Discussion

Regeneration of shoots and roots from explants of red banana **:** Male inflorescence and the bunch of red banana fruits are shown in Figure 1a and 1b respectively. From the field grown plants, suckers and male flower buds were used as explants for initial shoot regeneration on MS basal medium supplemented with 5 mg/L BAP plus 2 mg/L IBA. Shoots were rooted on MS medium containing 3 mg/L IBA. Plant regeneration frequency was found better from

male flower buds compared to suckers (data not shown). Thus, shoot regeneration was standardized before genetic transformation experiments were taken up.

Co-cultivation with Agrobacterium and sensitivity of explants to hygromycin: Recombinant vector was constructed with *P5CSF129A* gene driven by CaMV35S promoter and the map is shown in Figure 2. To optimize genetic transformation, experiments were performed with the choice of explant, pre-culture and co-cultivation period, effective density of *Agrobacterium*, acetosyringone concentration, and the effect of hygromycin on regeneration. Explant type and co-cultivation period influenced the transformation efficiency (Table 1). Male flower buds used for co-cultivation were found better for transformation compared to suckers and are shown in Figure 3a. Of different periods of co-cultivation (0-5 days), 3-day co-cultivation was found ideal. A 30-minute immersion in bacterial culture followed by a co-cultivation period of 3-days was optimal which resulted in significantly higher rate of GUS expression. Co-cultivation beyond 3-days led to bacterial overgrowth and resulted in the death of explants (Table 1). In the present study, a 5-day pre-culture of explants in the shoot induction medium was found critical to achieve high frequency transformation. After one week of culture, shoots were produced from male flower buds and suckers but 98% of them were escapes probably due to pre-emergence of shoot buds. When explants were co-cultivated without pre-culture, 90% of the explants were unable to withstand the infection and eventually died. Hence, in subsequent experiments, explants pre-cultured for 5-days on shoot induction medium were used. The period of agro infection and co-cultivation of explants with *Agrobacterium* is an important factor

Fig. 1.(a) Male inflorescence (b) Bunch of red banana fruits

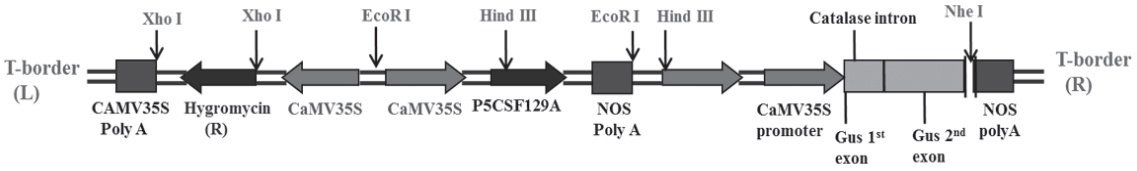

Fig. 2. Recombinant vector map of pCAMBIA1301-P5CSF129A gene construct.

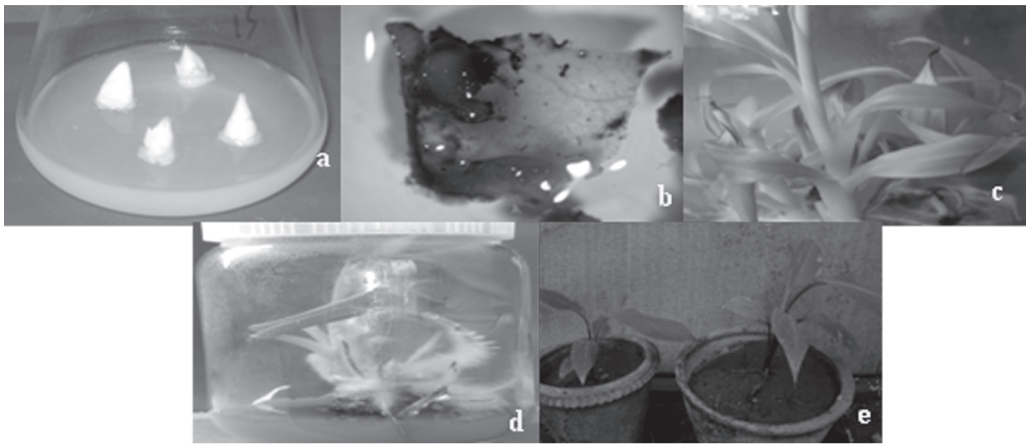

Fig. 3. Different stages of Agrobacterium - mediated transformation of red banana: (a) Excised male flower buds used for co-cultivation (b) Transgenic leaf tissue showing transient GUS expression (c) Shoots regenerated on MS medium containing 5 mg/L BAP + 2 mg/L IBA + 250 mg/L cefotaxime and 25 mg/L hygromycin (d) Root differentiation from transgenic shoots (e) Hardened transgenic plants growing in green house.

for efficient transformation as revealed in the present investigation. An immersion period of 30 min was optimal for agro infection, while 15 min was not sufficient and immersion for 40 min resulted in the overgrowth of bacteria. An inoculum density of 0.5 OD ($A_{600\ nm}$) showed high frequency of GUS activity. It was noticed that OD greater than 1 resulted in bacterial overgrowth. Transformation efficiency was 80% at 0.5 OD and densities lower and higher than this resulted in decreased transformation efficiency (data not shown). Addition of acetosyringone at different concentrations (0-120 µM) was found as an important factor for increasing the frequency of transformation. Among different concentrations of acetosyringone tested, 100 µM was found the best beyond which frequency of transformation and number of shoots formed per culture declined (Table 2).

***Selection of transformants and their molecular confirmation*:** In order tofind outthe appropriate concentration of the selection agent in red banana, different (0-25 mg/L) concentrations of hygromycin were employed during shoot bud production (Table 3). On medium without and with lower concentrations of hygromycin, luxuriant growth of shoots was observed. With an increase in the concentration of hygromycin to more than 5 mg/L, shoot bud induction was reduced from 100 to 60%, and at 25 mg/L, all the explants displayed necrosis and none of them survived after 8 days (Table 3). Three hundred explants inoculated on shoot induction medium supplemented with 25 mg/L hygromycin resulted in a transformation frequency of 18.66%. Transient expression of GUS activity was noticed in the transformants but not in untransformed controls (Fig. 3b). Shoots from male flower buds were regenerated (Fig. 3c) on MS medium fortified with 5 mg/L BAP + 2 mg/L IBA + 3% sucrose, 250 mg/L cefotaxime and 25 mg/L hygromycin (shoot induction medium). Well-developed shoots were excised and rooted on MS basal medium supplemented with 3 mg/L IBA and 25 mg/L hygromycin (Fig. 3d). Regenerated plants were first transferred to polycups and later into pots containing soil, sand and vermiculite (Fig. 3e). A total of 10 plants were transferred to pots for hardening, out of which 4 plants survived (data not shown). PCR analysis of putatively transformed (T_0) plants resulted in the detection of an amplification product of a 2.8 kb with gene specific primers corresponding to the coding region of *P5CSF129A* gene but the same was

not noticed in untransformed controls (Fig. 4). Amplification of *hptII* gene in transgenics using gene specific primers resulted in a band size of 750 bp (Fig. 5a). Southern blot analysis was performed for the two PCR positive lines, using genomic DNA isolated from leaf of T_0 and untransformed control plants. Genomic DNA was digested with *Eco RI*, followed by hybridization with the *P5CSF129A* probe, and as expected the whole cassette measuring 3.8 kb was observed in the two transgenic lines but no hybridization signal was detected in the untransformed control plants (Fig. 5b). In support of this, proline content was analyzed in untransformed controls as well as in T_0 plants, after one month of transfer of plants to the greenhouse. Two to three-fold increases in proline content were noticed without imposing any stress in the transgenic lines compared to controls (Fig. 6).

Efficient transformation and subsequent regeneration of plants, using *A. tumefaciens* mediated transformation are dependent on several factors such as type of explants, genotype, hormonal composition, duration of co-cultivation, duration of selection, type and concentration of antibiotic marker used for selection (25). In the present study, both suckers and male flower buds from red banana were used as explants for transformation. Different explants such as embryogenic cell suspensions (14, 26), male flower buds (15) and intercalary meristems (27) were used for *A. tumefaciens*-mediated transformation in banana. The superiority of male flower buds for transformation studies over that of suckers indicates that the explant type is important. Transgenic banana plants were reported earlier using *A. tumefaciens* mediated transformation (14, 17). The frequency of transformation was 18.66% in the present study compared to 35.1% as reported by Matsumoto *et al.* (28). This could be due to the differences in the genotypes used. In the present study, an inoculum density of 0.5 OD ($A_{600\ nm}$) was found ideal for co-cultivation of explants, OD greater than 1 resulted in bacterial overgrowth. Inoculum density corresponding to OD value varies widely

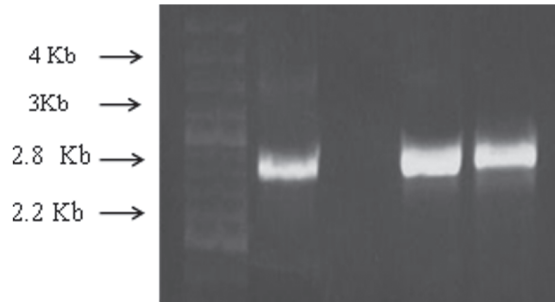

Fig. 4. PCR amplification of P5CS F129A gene using gene specific primers (T0 transformed plants): Lane M = 1 kb molecular weight marker; Lane +C = amplification of vector DNA; Lane C = untransformed control plants; Lanes 1 and 2 = putative transgenic T0 lines.

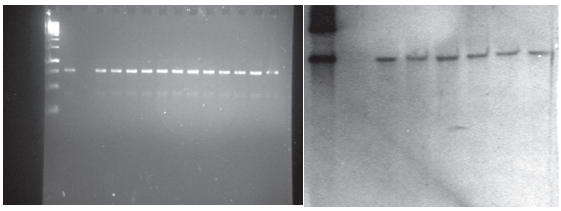

Fig. 5a and 5b. PCR amplification ofhpt II gene (750 bp) not observed in untransformed control but noticed in T0 transgenics (1 and 2): Fig. 5a-Lane M = Molecular weight marker (1 Kb ladder); Lane +C = Vector DNA, Lane C = untransformed control, Lanes 1 to 2 = T0 transgenics. Fig. 5b-Southern blot analysis of transgenic banana: Lane +C =vector control, Lane C = Untransformed control plant without insert, Lanes 1, 2 = Independent transgenics (1 and 2) showing P5CSF129A gene insert measuring 3.8 kb.

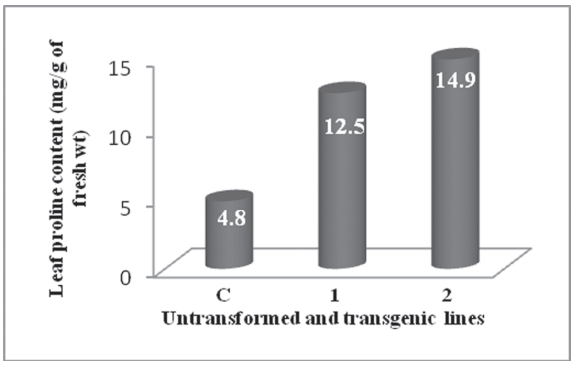

Fig. 6. Accumulation of proline in leaves of untransformed controls (C) and T0 transgenic lines (1 and 2) of red banana without any stress.

Table 1. Effect of co-cultivation period and explant type on transformation efficiency of red banana

Explant type	Co-cultivation period and transformation frequency*			
	2 Days	3 Days	4 Days	5 Days
Sucker	34.0 ± 0.98^{c}	50.4 ± 0.45^{a}	40.3 ± 0.35^{b}	30.0 ± 0.00^{d}
Male flower bud	45. 0 ± 0.00^{c}	70.0 ± 1.50^{a}	65.4 ± 0.50^{b}	40.2 ± 0.33^{d}

*MS medium supplement with 5 mg/L BAP + 2 mg/L IBA was used for shoot regeneration. Data represent average of three replicates; each replicate consists of 25 cultures. Mean ± standard error, Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

Table 2. Effect of acetosyringone concentration on transformation frequency

Concentration of acetosyringone* (µM/L)	Explants			
	Male flower bud		Sucker	
	Frequency (%)	No. of shoots/culture	Frequency (%)	No. of shoots/culture
00	00	0.0 ± 0.00	00	0.00 ± 0.00
20	30	6.0 ± 0.50^{e}	20	3.0 ± 0.00^{e}
40	30	9.4 ± 0.45^{d}	20	7.0 ± 0.00^{d}
60	70	11.0 ± 0.86^{c}	50	9.4 ± 0.00^{c}
80	90	14.0 ± 0.56^{b}	70	11.8 ± 0.00^{b}
100	100	20.7 ± 0.34^{a}	90	15.3 ± 0.00^{a}
120	80	12.0 ± 0.50^{c}	70	10.1 ± 0.00^{c}

*MS medium supplement with 5 mg/L BAP + 2 mg/L IBA was used for shoot differentiation. Data represent average of three replicates; each replicate consists of 25 cultures. Mean ± Standard error, Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

Table 3. Frequency of shoot regeneration, rooting and survival of plants after gene transfer through*Agrobacterium*

Concentration of acetosyringone* (µM/L)	Explants			
	Male flower bud		Sucker	
	Frequency (%)	No. of shoots/culture	Frequency (%)	No. of shoots/culture
0	100	30.6 ± 0.34^{a}	100	14.8 ± 0.21^{a}
05	80	20.7 ± 0.25^{b}	60	11.4 ± 0.25^{b}
10	60	14.0 ± 0.33^{c}	40	8.0 ± 0.00^{c}
15	50	7.8 ± 0.50^{d}	10	6.4 ± 0.25^{d}
20	30	4.0 ± 0.00^{e}	00	1.0 ± 0.00^{e}
25	0	0.0 ± 00	0	0.0 ± 00

*Data represent average of three replicates; each replicate consists of 25 cultures. Mean ± standard error, Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

in banana. While Matsumoto *et al.* (28) reported an OD value of 0.4, Maziah *et al.(*27) noticed that an inoculum density of 0.7 OD as optimum for transformation in banana cv. Rasthali (AAB genome). Likewise, Huang *et al.* (26) and Tripathi *et al.* (29) reported 0.8 OD for efficient transformation in the cv Mass (AA) and East African highland banana respectively. The above results clearly show that inoculum density of bacterial cultures vary from cultivar to cultivar. Co-cultivation duration for banana transformation has been reported to vary between 2-5 days. In this study, it was found that a co-cultivation period of 3-days was suitable for red banana transformation. Earlier, Ganapathiet *et al.* (14), Tripathi *et al.* (16) and Sreeramanan *et al.* (30) also reported a 3-day co-cultivation period for high frequency transformation in banana. In the present study, 25 mg/L hygromycin was found suitable for selecting the putative transformants. Our results are in agreement with the results of Tripathiet *et al.* (29). Plant specific phenolic compounds that induce the expression of *vir*genes are important for the gene transfer (31). Acetosyringone has been reported to enhance the frequency of transformation both in dicots (12) and monocots (32). Addition of acetosyringone to the co-cultivation medium has been reported to induce *vir* genes, which extends the host range of some *Agrobacterium* strains and found most essential for banana transformation (27). Our observations are in confirmation with the findings of Tripathiet *et al.* (16) who reported that inclusion of 100 µM acetosyringone was inevitable for transformation. However, Matsumoto *et al.* (28) reported that 200 mM acetosyringone was required for Nanicao and CNPMFF varieties of banana for high frequency transformation. This difference in the requirement of different concentrations of acetosyringone may be due to varietal differences. Transformed T_o plants displayed GUS expression in leaf tissues but the same was not observed in the untransformed controls. These findings are in agreement with the reports of others (14, 29, 26). These results establish that *A. tumefaciens* can be employed for stable genetic transformation in red banana.

Transgenics showed elevated levels of proline (2 to 3-folds) under non-stressed conditions compared to the untransformed plants. This clearly confirmed the functional expression of *P5CSF129A* gene in the banana genome. The first plant to be transformed with *P5CS* gene was that of tobacco, which showed elevated levels of proline (5). Similar to our results, the primary *P5CSF129A* – transgenic wheat plants displayed more than ten times proline content than their wild type counterparts without any stress (33). Recently, Yamchiet *et al.* (11) observed that there was a 26-times more proline production in tobacco plants transformed with *P5CSF129A* gene as compared to the untransformed controls. Kumar *et al.* (34) also reported high proline content in rice transformed with *P5CSF129A* gene. Introduction of *P5CSF129A* gene is shown to confirm abiotic stress tolerance due to elevated levels of proline in several plants like rice (34), wheat (33), carrot (7), chickpea (12) and pigeonpea (35). However; abiotic stress tolerance has not been carried out in the present study in red banana. Successful insertion of *P5CSF129A* gene has been achieved in the T_0 generation of red banana as revealed by PCR and Southern analysis. The gene was functionally expressed as indicated by elevated levels of proline accumulation in transgenics as compared to the untransformed controls. It is concluded that male flower bud is the ideal explant for genetic transformation of red banana. Thus, this work paves the way for developing transgenic red banana with improved agronomic traits.

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References

1. Rengasamy, P. (2006). World salinization with emphasis on Australia. J. Exp. Bot., 57: 1017-1023.

2. Greenway, H and Munns, R. (1980). Mechanisms of salt tolerance in non-halophytes. Annu Rev Plant Physiol.,31: 149-190.
3. Kavi Kishor, P.B., Sangam, S., Amrutha, R.N., Sri Laxmi, Naidu, K.R., Srinath Rao, Reddy, K.J., Theriappan P. and Sreenivasulu N. (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Curr Sci., 88: 422-443.
4. Hare, P. and Cress W. (1997). Metabolic implications of stress induced proline accumulation in plants. Plant Growth Reg., 21: 79-102.
5. Kavi Kishor, P.B., Hong, Z., Miao, G., Hu, C.A.A. and Verma, D.P.S. (1995). Overexpression of Ä[1]-pyrroline-5-carboxylatesynthetase increases proline production and confers osmotolerance in transgenic plants.Plant Physiol., 108: 1387-1394.
6. Zhu, B., Su, J., Chang M., Verma, D.P.S., Fan, Y.L. and Wu, R. (1998). Overexpression of a Ä[1]-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water and salt-stress in transgenic rice. Plant Sci.,139: 41-48.
7. Han, K.H., and Hwang, C.H. (2003). Salt tolerance enhanced by transformation *P5CS* gene in carrot.J Plant Biotechnol., 5: 149-153.
8. Schobert, B. and Tschesche, H. (1978). Unusual solution properties of proline and its interaction with proteins. Biochim BiophysActa., 541: 270-277.
9. Smirnoff, N. and Cumbes, Q.J. (1989). Hydroxyl radical scavenging activity of compatible solutes. Phytochem.,28: 1057-1060.
10. Hu, C.A.A., Delauney, A.J. and Verma, D.P.S. (1992). A bifunctional enzyme (Ä[1]-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proc Natl Acad Sci, USA., 89: 9354-9358.
11. Yamchi, A, Jazii F.R, Mousav A, Karkhane A. and Renu, A. (2007). Proline accumulation in transgenic tobacco as a result of expression of *Arabidopsis* Ä[1]-pyrroline-5-carboxylate synthetase (P5CS) during osmotic stress.J Plant Biochem Biotechnol., 16: 9-15.
12. Ghanti, S.K., Sujata, K.G., Vijay Kumar, B.M., Nataraja Kabba, N., Janardhan Reddy, K., SrinathRao, M. and Kavi Kishor, P.B. (2011). Heterologous expression of *P5CS* gene in chickpea enhances salt tolerancewithout affecting yield. Biologia Plant., 55: 634-640.
13. Singh, H.P, Uma, S, Selvarajan, R. and Karihaloo, J.L. (2011).Micropropagation for production of quality banana planting material in Asia-Pacific. Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB), New Delhi.
14. Ganapathi, T.R., Higgs, N.S, Balint-Kurti, P.J. and Van-Eck J. (2001).*Agrobacterium*-mediated transformation of embryogenic cell suspensions of banana cultivar Rasthali (AAB). Plant Cell Rep., 20: 157-162.
15. Khanna, H., Becker D., Kleidon J. and Dale, J. (2004). Centrifugation assisted *Agrobacterium tumefaciens*-mediated transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. Cavendish AAA and Lady Finger AAB). MolBreed., 14: 239-252.
16. Tripathi, L, Tripathi, J.N. and Hughes, J.D.A. (2005). *Agrobacterium* mediated transformation of plantain *(Musa*sps) CvAghagba. Afr J Biotech., 4: 1378-1383.
17. Sreeramanan, S., Mazia, M., Abdullah,M.B. and Saria Xanier, R. (2006). Transient expression of GUS and *gfp* gene in *Agrobacterium*-mediated banana transformation using single tiny meristematic bud. Asian J Plant Sci., 1: 1-13.
18. Sagi, L., Panis, B., Remy, S., Schoofs, H., Smet, K.D., Swennen, R. and Cammue, B.P.A. (1995). Genetic transformation of

banana and plantain (*Musa* spp.) via particle bombardment.Biotechnology., 13: 481-485.

19. Becker, D.K., Dugdale, B., Smith, M.K., Harding, R.M. and Dale, J.L. (2000). Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv Grand Neyne via microprojectile bombardment. Plant Cell Rep., 19: 229-234.
20. Murashige, T. and Skoog, F.A. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant., 15: 473-497.
21. Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987).*GUS*-fusions: â-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J.,6: 3901-3907.
22. Doyle, J. and Doyle, J. (1990). Isolation of plant DNA from fresh tissue. Focus.,12: 13-15.
23. Sambrook, J. and Russell, R.W. (2001). Molecular cloning: A laboratory manual. Cold SpringHarbour Laboratory Press, Cold Spring Harbour, USA.
24. Bates, L.S, Waldren R.P. and Teare, I.D. (1973). Rapid determination of free proline for water stress studies. Plant Soil., 39: 295-208.
25. Indurker, S., Misra H.S. and Eapen E. (2010). *Agrobacterium*-mediated transformation in chickpea (*Cicerarietinum* L.) with an insecticidal protein gene: optimization of different factors. Physiol MolBiolPlants., 16: 273-284.
26. Huang, X, Huan L X., Xiow W., Dai X M., Chen Y.F. and Li, X.J. (2007). Highly efficient *Agrobacterium* mediated transformation of embryogenic cell suspension of *Musa acuminata*cv Mas (AA) via a liquid co-cultivation system.Plant Cell Rep., 26: 1755-1762.
27. Maziah, M., Sreeramanan, S.P., Uad A. and Sariah, I. (2007). Production of transgenic banana cultivar Rasthali (AAA) via *Agrobacterium* mediated transformation with rice chitinase gene. J Plant Sci., 2: 501-517.
28. Matsumoto, K., Cabral G.B., Batista-Teixeira, J. and Castro Monte, D. (2009). *Agrobacterium*-mediated transient expression system in banana immature fruits.Afr J Biotech.,8: 4039-4044.
29. Tripathi, L., Jaindranath T. and Fateera W.T. (2008). Rapid and efficient production of transgenic east African island banana (*Musa*. sps) using intercalary meristematic tissue. Afr J Biotech., 7: 1438-1445.
30. Sreeramanan, S., Maziah, M. and Xavier, R. (2009). A protocol for *Agrobacterium*-mediated transformation of banana with a rice chitinase gene. Emir J Food Agric., 21: 18-33.
31. Stachel, S.E., Messens M., Van-Montagu, P. and Zambryski, P. (1985). Identification of the signal molecules produced by wounded plant cells which activates the T-DNA transfer process in *Agrobacterium tumefaciens*. Nature, 318: 624-629.
32. Rashid, H., Chaudhry, Z. and Khan, M.H. (2011). Effect of explant plant source and acetosyringone concentration on transformation efficiency of wheat cultivars. Afr J Biotech., 10: 8737-8740.
33. Sawahel, W.A. and Hassan, A.H. (2002). Generation of transgenic wheat plants producing high levels of the osmo-protectantproline. Biotech Lett., 24: 721-72.
34. Kumar, V., VarshaShriram, KaviKishor P.B., Jawali, N. and Shitole, M.G. (2009). Enhanced proline accumulation and salt stress tolerance of transgenic*indica* rice by over-expressing *P5CSF129A* gene. Plant Biotech Rep., 4: 37-48.
35. Surekha, C., Nirmala Kumari, K., Aruna, L.V., Suneetha, G., Arundhati, A. and Kavi Kishor, P.B. (2014).Expression of the *Vignaaconitifolia P5CSF129A* gene in transgenic pigeonpea enhances proline accumulation and salt tolerance. Plant Cell Tiss Organ Cult., 116: 27-36.

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Intervertebral Disc Proteome Database in Human (TNAUPDB –Human Proteome)

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Abstract

Human intervertebral disc (IVD) proteome is an open accessible database of Tamil Nadu Agricultural University Proteome DataBase (TNAU PDB) that focuses on proteome of human intervertebral disc. Currently, the database contains reference maps of Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) of proteins obtained from degenerated intervertebral disc. The initial 2D-PAGE and MALDI TOF studies have revealed that abundantly expressed 30 protein spots. The database provides information about experimentally identified properties, such as molecular weight, pI value and protein identities obtained using MALDI-TOF and MASCOT analysis. This database runs on WAMP server with HTML as the front end and MySQL as the backend using PHP as interface and it is hosted in TNAU genomics domain. The basic intention of this database is to provide information about intervertebral disc proteome. Further information on differentially expressed proteins on comparison with healthy and degenerated intervertebral disc tissue will help us to gain insights into the underlying molecular mechanism in disc degenerative disease. This database will be a valuable biological resource for the treatment of disc degenerative disease.

Availability: The human intervertebral disc proteome database is freely available at http://www.tnaugenomics.com.

Keywords: Intervertebral disc, Disc degenerative disease, database, proteomics, proteome database, TNAUPDB, TNAU genomics.

Introduction

Low back pain (LBP) is a highly prevalent musculoskeletal disorder in mankind, caused by degeneration of intervertebral discs (IVD) of the lumbar spine (1). It is one of the leading causes of disability in the industrialized world including India. The prevalence of low back pain in the society is reported to be around 60 per cent and 80 per cent of the population suffers at least one episode of back pain in their life (2). The annual prevalence ranges from 15 to 45% with the point prevalence averaging 30% (3). The costs associated with this condition are enormous, including both direct medical costs and indirect costs, such as decreased productivity in the workplace. LBP is therefore not only a health problem but also a socio-economic problem. The second most frequent reason for visits to physicians, the fifth-ranking cause of admission to hospital and the third most common reason for surgical procedures (4).

Molecular mechanism(s) underlying LBP/DDD is still unclear mainly because of the difficulty in obtaining intact IVD tissue from patients for molecular studies. So far, only a very few attempts have been made to understand the molecular basis of DDD in humans. Recent reports are showing enough evidences to the involvement of genetic factors and a number of

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genes.However, functional studies of candidate genes will be an important step for testing whether a candidate gene is truly associated with DDD or not. Hence proteomic approach will be a better option to understand the molecular mechanism of disc degenerative disease.

Methodology

Database Content and Source *:* Proteins were extracted from the degenerated IVD was obtained from a fifty two year old female who underwent spine surgery for lumbar disc prolapse after getting permission from Ethical Clearance Committee. IVD tissue sample was pulverized using liquid nitrogen and proteins were extracted as explained by Belluoccio *et al.* (2006) with minor modifications. Extracted proteins from the single patient were subjected to 2D-PAGE analysis (Figure 1) and abundantly expressed proteins were analyzed using MALDI TOF analysis, and the putative functions of these proteins were annotated by MASCOT analysis. Hence, currently, the TNAUPDB human intervertebral disc proteome database consists of information on abundantly expressed proteins from degenerated intervertebral disc tissue.

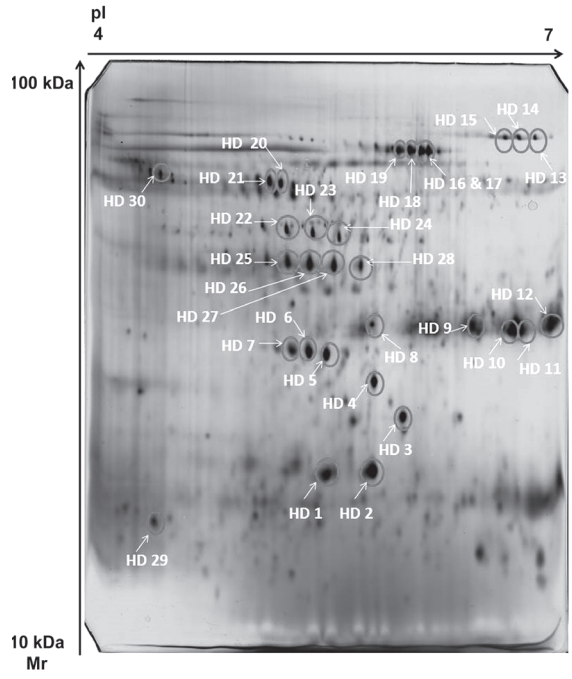

Fig. 1. 2D PAGE protein profiles of intervertebral disc tissue

Languages and softwares used *:* The front end of the web application is developed on HTML 5.0 (Hyper Text Markup Language) and the validations are done using javascript. The server side scripting was done on PHP 5.4 (Hypertext Pre Processor) and the application was connected to the database using MySQL 5.5.16. PhpMyAdmin provides a graphical user interface for the MySQL database. Web application was created by using wamp server 2.3 windows web development environment. Adobe Dreamweaver was used to link each spot in the gel image to the corresponding protein information.

Techniques behind the database *:* In order to provide effective information from biological data, advanced web query interface is created. Web query interface was created using HTML as the front end and MySQL as the back end with Hypertext preprocessor (PHP) as the interface. Database indexing is a method that is used to improve the speed of data retrieval operations on database table. Clustering method was used to retrieve group of similar object based on the maximum and minimum value. Data are stored as relational model database (5).

Database architecture *:* This human intervertebral disc database architecture is accumulated in three layers similar to black gram proteome database (6). Presentation layer, Application layer and Data storage layer as shown in the Figure 2. The database is built on a three layer architecture model consisting of presentation layer, application layer and the data storage layer. The presentation layer or the top most layer is created using HTML which serves as interface for the user to interact with the database. The middle layer or the application layer is created using PHP, which connects the user interface with that of the database. This application layer transfers the user's query to the database and retrieves the result from it. The

Fig. 2. Database architecture

bottom layer or the data storage layer (MySQL) contains the data tables from which the database results are retrieved.

Results and Discussion

Data flow *:* Intervertebral disc proteome database has been added as a sub link under TNAU genomics domain which has home page under the link http://www.tnaugenomics.com. From the homepage, it can be reached through the drop down link named TNAUPDB under the tab Databases. The flow of data and the procedure to access the database is shown in Figure 3.

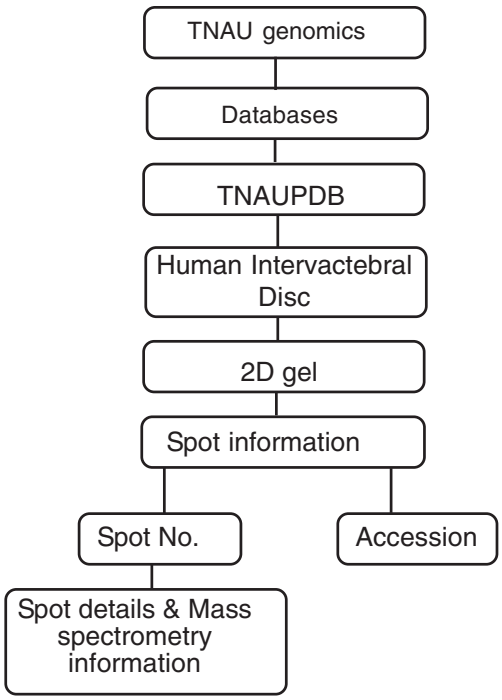

Fig. 3. Data flow

Database Schema *:* The database contains the protein information from intervertebral disc. Here, the 30 abundantly expressed IVD proteins identified using the 2D PAGE and MALDI-TOF (Table 1) are displayed using image analyzer software and the description of each spot are mapped to the corresponding spots using Adobe Dreamweaver. The reference 2D-PAGE gel shows the position of each identified protein. By selecting the spot number in the gel image, the entire information about that protein such as spot ID, molecular weight, isoelectric point, organism, tissue, homologous protein name, sequence coverage, theoretical molecular weight and isoelectric point, mascot score and mascot result can be obtained. The experimental protocols are listed and are freely available under the protocol section. Some of the major proteomics tools like Mascot, Compute pI/Mw tool in ExPASy and ExPASy Proteomics tools are displayed under proteome tools section. Similar advanced proteome databases are available for Arabidopsis and maize by Cornell university (7) and DynaProt 2D for dynamic online access to proteomes and two-dimensional electrophoresis gels (8).

Utility of the intervertebral disc proteome database to the biological community : Proteomics is one of the high throughput technologies in post genomics era. In the database, we have provided a snapshot of human IVD proteome. Further studies are required to dissect the functions of these differentially expressed proteins from both control

Table 1. Identification of IVD proteins by MASCOT analysis

Spot ID [1]	Coverage [2]	Accession No [3]
HD1	48	167887751
	25	19908424
HD2	31	205360981
HD3	11	119619469
HD4	47	914833
HD5	64	90108665
HD6	49	90108664
HD7	30	62088402
HD8	61	159163872
HD9	27	119612556
	27	4929613
HD10	37	3721836
HD11	34	134254718
HD12	30	3660517
HD13	33	296676
HD14	48	194387990
HD15	21	119612556
HD16	27	31455194
HD17	61	145938571
HD18	44	40788217
HD19	28	119592961
HD20	54	110624781
HD21	64	159163872
HD22	42	332245896
	43	13543925
HD23	63	39653325
HD24	25	121934188
HD25	26	385198093
HD26	64	3660517
HD27	27	385198093
HD28	39	4867999
HD29	27	385198093
HD30	14	17426164

[1] ID refers to the corresponding spot number in fig. 1

[2] Amino acid sequence coverage

[3]Corresponding accession numbers for the identified proteins were obtained from NCBI (www. ncbi. nlm.nih.gov).

and degenerated disc to understand their precise role in the disc biology and hence pave way for designing novel strategies to treat the disc degenerative disease. The primary users/ beneficiaries of this database will be spine surgeons, biochemist, students and other research scholars interested in treatment of disc degenerative disease.

Future developments : The intervertebral disc proteome database will be updated regularly. The scientists of TNAU Genomics and Proteomics lab will deposit and update their new data that become accessible on the web. In future, data on control IVD proteome and data on differentially expressed protein also will be made available.

Conclusion

TNAU PDB – Intervertebral disc Proteome database focuses on proteome of human IVD. Currently, data on degenerated IVD tissue proteome is made available, and the database will be updated routinely as and when new data are obtained. Hence, this database will be highly helpful to spine surgeons, biochemist, students and other researcher scholars interested in treatment of disc degenerative disease.

References

1. Solovieva, S., Kouhia, S., Leino-Arjas, P., Ala-Kokko, L., Luoma, K., Raininko, R., Saarela, J., Riihimäki, H. (2004). Interleukin 1 polymorphisms and intervertebral disc degeneration. Epidemiology, 15(5):626-633.

2. Seki, S., Kawaguchi, Y., Chiba, K., Mikami, Y., Kizawa, H., Oya, T., Mio, F., Mori, M., Miyamoto, Y. and Masuda, I. (2005). A functional SNP in CILP, encoding cartilage intermediate layer protein, is associated with susceptibility to lumbar disc disease. Nature genetics, 37(6):607-612.

3. Andersson, G.B. (1999). Epidemiological features of chronic low-back pain. The lancet, 354(9178):581-585.

4. Hart, L.G., Deyo, R.A., Cherkin, D.C. (1995). Physician office visits for low back pain: frequency, clinical evaluation, and treatment patterns from a US national survey. Spine, 20(1):11-19.

5. Murukarthick, J., Senthil, N., Raveendran, M., Prabhakaran, P., Sreedevi, G., Sumanth Kumar, M., Shobhana, V., Dhanya, S., Khushboo, R., Arumugasamy, S. (2011). Biogen Base–An Interactive Maize Database for Phenomics Platform. International Journal of Computer Application, 2:56-61.

6. Swathi, S., Senthil, N., Kumar, V.V., Sathish, S., Selvam, N.J., Raveendran, M. (2013). TNAU PDB-Tamil Nadu Agricultural University Proteome DataBase-Black Gram Proteome. American Journal of Experimental Agriculture, 3(4):971-976.

7. Sun, Q., Zybailov, B., Majeran, W., Friso, G., Olinares, P.D.B., van Wijk, K.J. (2009). PPDB, the plant proteomics database at Cornell. Nucleic acids research, 37(suppl 1):D969-D974.

8. Drews, O. and Görg, A. (2005). DynaProt 2D: an advanced proteomic database for dynamic online access to proteomes and two-dimensional electrophoresis gels. Nucleic acids research, 33(suppl 1):D583-D587.

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Role of TCA cycle Truncation in Cancer Cell Energetics

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Abstract

Continuously dividing cancerous cell requires duplication of its genome and other cellular building blocks before every cell division. This increased demand for amino acids, nucleotides and fatty acids, require readjustment of metabolic pathways, specially glycolysis and TCA cycle, to divert intermediates of these series of reactions from energy generating process (OXPHOS) to synthesis of cellular macromolecule building blocks. This altered cellular metabolism has been observed as early as 1926. Otto Warburg observed aerobic glycolysis and proposed defective mitochondria as a cause of cancer. Since then, 'Warburg effect' has become a hallmark of cancer detection by positron emission tomography (PET) and is now being perused for designing of novel anticancer drug targeting enzymes like PKM2. However, since beginning, Warburg hypothesis of nonfunctional mitochondria has been questioned. Now it is clear that cancerous cell has intact and functional mitochondria, moreover reverse. Warburg effect has proposed a nonfunctional mitochondria in stromal tissue and with functional OXPHOS system for ATP synthesis in cancerous cells. These two hypotheses of Warburg and reverse Warburg show two extremes one relying almost completely on aerobic glycolysis and another on OXPHOS for ATP synthesis in cancerous cell, but both of them fail to explain how cell readjusts to meet increased demand for ATP synthesis as well as cellular building blocks simultaneously, from the intermediates of glycolysis and TCA cycle. In the present hypothesis, we present how truncation of TCA cycle can increase rate of synthesis of amino acids and simultaneously TCA cycle gets completed to generate NADH and FADH2. Oncomine database analysis further shows upregulation of ATP synthase genes and indicative of increased OXPHOS I cancerous cell. In this background, we propose that cancerous cell utilizes both aerobic glycolysis and OXPHOS, for synthesis of ATP and to meet increased requirement of proteins, nucleic acids and fatty acids.

Key words: Metabolic rearrangement, onco genomics, oncogenic mutations, TCA cycle, ROS

Introduction

A common feature across most of cancer types is increased glucose uptake and utilization. In contrast to normal cells, cancerous cell convert glucose to lactate even under aerobic conditions (1). Direct relationship between malignant transformation and increased glucose consumption has been nicely demonstrated by increased glucose transport rate in cancer cell lines. This fact is widely exploited for detection of cancer by ^{18}F-deoxyglucose positron emission tomography (FDG-PET) (2, 3). Warburg proposed that aerobic glycolysis in cancer is the result of defective mitochondria (4-7) which leads to failure of energy generation by oxidative phosphorylation. The irrefutable facts that (i)

cancer cells consume more glucose in comparison to normal cell, (ii) secrete high concentration of lactate and (iii) when supplemented with radio labeled glucose as carbon source most of the radioactivity is traced to lactate rather than tricarboxylic acid cycle (TCA cycle) intermediates supports 'Warburg Effect'. The adoption of an inefficient system of energy generation (2 ATP *vs* 36 ATP) in highly proliferating cancerous cells is an enigma since long. However, the existence of inefficient aerobic glycolysis in high energy demanding tissues (skeletal muscle), brain and rapidly dividing cells corroborates this inefficient aerobic glycolysis in actively proliferating cancerous cells (8, 9).

Warburg's hypothesis does not fully justify the growth behavior of cancer cells for e.g., there is not much reduction in oxygen consumption in cancerous cells. Further research over the years has clearly revealed that mitochondria in cancerous cells are intact and cancerous cell utilizes high dose of glutamine in addition to glucose (10-12).The major challenge to this theory has come in the last five years, when one research group showed that mitochondrial dysfunction with non-functional OXPHOS and activated aerobic glycolysis is in fact observed in the stromal tissue surrounding the cancerous cells but not in cancerous cells as proposed by Warburg (13). In this new hypothesis termed as 'Reverse Warburg' it is reported that mitophagy induces autophagy in stromal tissue followed by the nutrient transportation from the stromal cells to cancerous cells (14-16). In this model, cancerous cells give autophagy signals to stromal cells and stromal cells provide nutrients to parasitic cancerous cells. Using various localization and functional assays, intact mitochondrial membrane and various functional enzymes from glycolysis, TCA cycle and ETC chain have been shown (17). According to 'Reverse Warburg' hypothesis, FDG-PET actually detects accumulation of radioactivity in stromal tissue, not in cancerous cells (14). These new findings have ultimately lead to 'neo-Warburg' hypothesis, which suggests that entry of glutamine in TCA cycle through alpha-ketoglutarate provides the much needed metabolic intermediates for biosynthesis of amino acids, nucleic acids and fatty acids (18). However, it is still assumed by proponents of 'Warburg' hypothesis that in a cancerous cell most of the energy comes from aerobic glycolysis. Thus, these two hypothesis depict two extremes- one the 'Warburg', shows reliance of cancerous cells on aerobic glycolysis and another the 'Reverse Warburg' relies entirely on OXPHOS and supply of nutrients from surrounding cancer cell microenvironment.

Cancer cell metabolism has been recently extensively reviewed, emphasizing on mutations and oncogenic signaling (19), OXPHOS in cancerous cells (20), aerobic glycolysis as drug target (21, 22), decoupling glycolysis and TCA cycle (23), regulation of different biochemical pathways (24), extra metabolic roles of TCA cycle metabolites (25) and signaling and communications with stromal cells (26). This review primarily summarizes various metabolic rearrangements in TCA cycle that are acquired to support the demand of energy (through OXPHOS and aerobic glycolysis) and synthesis of macromolecules in growing cancerous cells. Inferences from literature suggest that aerobic glycolysis and truncation of TCA cycle due to various physiological and genetic factors support increased biosynthesis of cellular building blocks. Truncated TCA cycle is compensated by activation of various alternative carbon (glutamate) utilization pathways and helps completion of truncated TCA cycle and generation of $FADH_2$ and $NADPH_2$ for synthesis of ATP through OXPHOS. Various structural adaptations in mitochondria, perhaps to incorporate more of ATP synthase, further support active participation of both aerobic glycolysis and TCA in tumorigenesis.

Truncation of TCA cycle and switch to multiple carbon sources in cancerous cells *:* The fact that continuous proliferation of cancerous cells (or cell lines) requires supply of glutamine (in addition to glucose) is widely

accepted by proponents of both 'Warburg' as well as 'Reverse Warburg' hypothesis. Probably the parallel consumption of two carbon sources comply with enhanced energy requirements and scale up synthesis of metabolic intermediates required for cell duplication. However, switch over to glutamate as carbon source provides a challenge for the cell. In a cell, glutamate to alpha-ketoglutarate conversion is a near thermodynamic equilibrium reaction, the net reaction can move either in or out of the Krebs's cycle, depending on the relative concentrations of the two on each side of the chemical equation (cataplerotic or anaplerotic) (27). Therefore, as long as enough glucose derived alpha-ketoglutarate is getting synthesized, glutamate cannot get utilized in the TCA cycle. Thus, in order to utilize glutamate in TCA cycle, synthesis of alpha- ketoglutarate from glucose derived carbon must get reduced/ stopped. This truncated TCA cycle actually leads to secretion of accumulated citrate out of mitochondria that gets utilized in nitrogen metabolism of cell. This also explains the observed high level of citrate secreted from mitochondria to cytosol (28) and aerobic glycolysis in cancers (12).

The question arises, how TCA cycle gets truncated in a cancerous cell, which has been following normal series of biochemical reactions from glycolysis, TCA cycle to ETC for energy production, before becoming cancerous? Apparently, the synthesis of alpha-ketoglutarate could be reduced or stopped either by inhibition or mutation in key enzyme like aconitase, iso-citrate dehydrogenase working upstream to alpha-ketoglutarate in TCA cycle. Further pyruvate kinase catalyzing last and irreversible step of glycolysis can truncate TCA cycle by limiting the supply of pyruvate (29) (Figure 1).

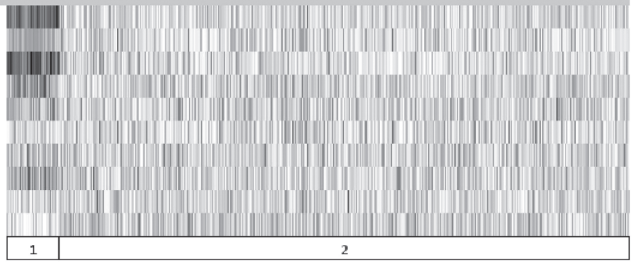
Pyruvate Kinase : Pyruvate kinase is a key enzyme that can truncate TCA cycle just at the entry point of pyruvate. Reduced pyruvate kinase activity enables the upstream glycolytic intermediates to accumulate and contribute to the shift of metabolism towards the anabolic phase for synthesis of amino acids and nucleotides. Cancer cells meet this increased demand by predominantly using PKM2, an isoform of pyruvate kinase, as its activity can be dynamically regulated between the less active PKM2 dimer and the highly active PKM2 tetramer (29, 30). PKM2 activity is allosterically regulated by serine which is synthesized from 3-phosphoglycerate, a glycolytic intermediate (31). Availability of serine activates human PKM2 while deprivation reduces its activity significantly (31). Thus, in such scenario, it seems that when demand of amino acids is more for protein synthesis, serine level gets depleted and in absence of activator serine, PKM2 fails to produce enough pyruvate to feed TCA cycle for energy production as well as for synthesis of other amino acids and fatty acids, starting from TCA intermediates (Figure 1 A). In case of PKM2 inhibition, conversion of glutamate to alpha-ketoglutarate not only complete the TCA cycle by subsequent conversion to succinate and fumarate but also get converted to citrate through carboxylation reaction (32).

Aconitase : Aconitase is another key enzyme that has potential to truncate TCA cycle, before synthesis of alpha-ketoglutarate. Aconitase enzyme that catalyses the stereo-specific isomerization of citrate to isocitrate is sensitive to inhibition by reactive oxygen species (ROS). Increase in ROS in cancerous cell is well known (33, 34) an its inhibition results in truncation of TCA cycle at just before entry point of glutamate (Figure 1B). This inhibition of aconitase by ROS and subsequent truncation of TCA cycle is also apparent in diabetic neuropathy (35). Apparently, the TCA cycle truncated either by inhibition of aconitase or PKM2 results in decreased alpha-ketoglutarate level. This triggers the activation of an anaplerotic conversion of glutamate to alpha-ketoglutarate and accumulation of various intermediates of TCA cycle. Subsequently, these get utilized in biosynthesis of fatty acids, nucleotides and amino acids. In fact, this increase in concentration of TCA metabolites and amino acid biosynthesis has been observed in aconitase mutant in *Arabidopsis* (36).

Supplementary data: Oncomine analysis of expression of Glycolysis, TCA cycle and ETC genes in different 1. Normal and 2. Breast cancer samples in a study by Curtis, et al. 2012 (45)

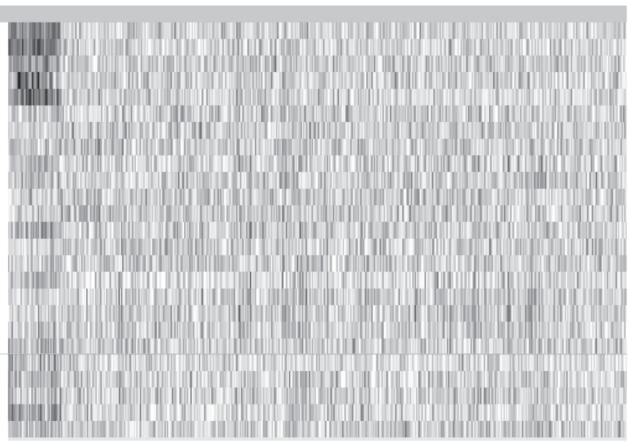
Glycolysis genes

P-value	Fold Change	Gene
4.38E-99	2.40	GAPDH
2.98E-98	1.71	TPI1
3.73E-70	1.85	HK1
3.50E-56	1.66	PGK1
1.13E-30	1.39	ENO1
7.70E-25	1.31	PGAM1
7.94E-20	1.22	PFKL
8.15E-13	1.34	GPI
1.000	-1.03	PKLR
1.000	-1.09	ALDOB



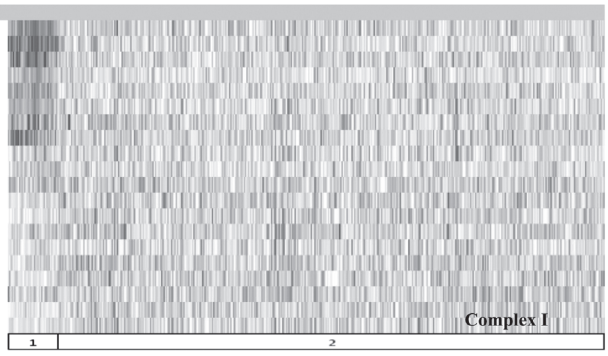
TCA cycle genes

P-value	Fold Change	Gene
2.44E-80	2.53	IDH2
4.04E-67	1.61	MDH2
1.28E-50	1.54	SDHC
2.49E-41	1.81	FH
1.68E-32	1.79	PCK2
1.83E-22	1.18	IDH3G
7.71E-19	1.14	IDH3B
1.49E-16	1.23	ACLY
6.60E-13	1.16	SDHB
5.37E-4	1.03	PC
0.001	1.08	OGDH
0.093	1.04	SUCLG2
0.125	1.04	ACO2
0.950	-1.04	SDHA
0.967	-1.05	IDH3A
0.981	-1.08	MDH1
0.999	-1.14	IDH1
1.000	-1.16	SDHD
1.000	-1.20	DLD
1.000	-1.17	SUCLG1
1.000	-1.16	PCK1
1.000	-1.31	CS
1.000	-1.36	SUCLA2
1.000	-2.28	ACO1
1.000	-1.33	DLST

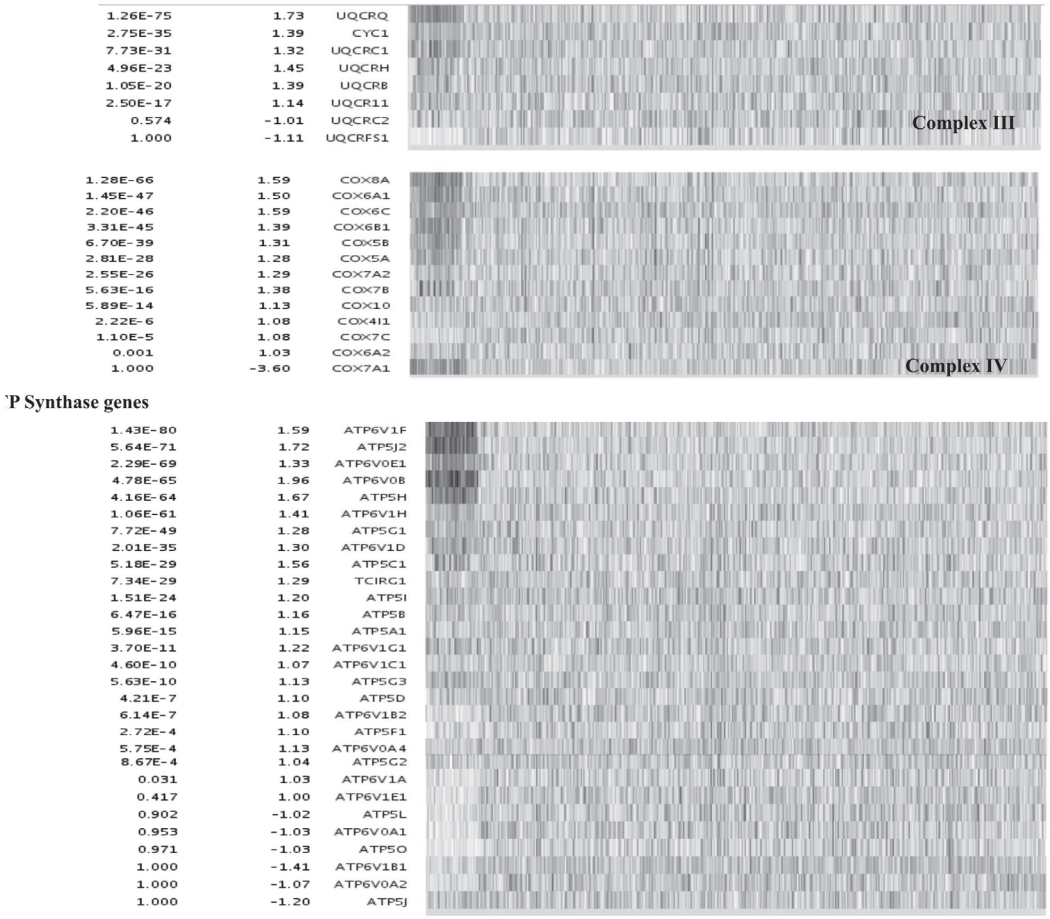


Electron transport chain

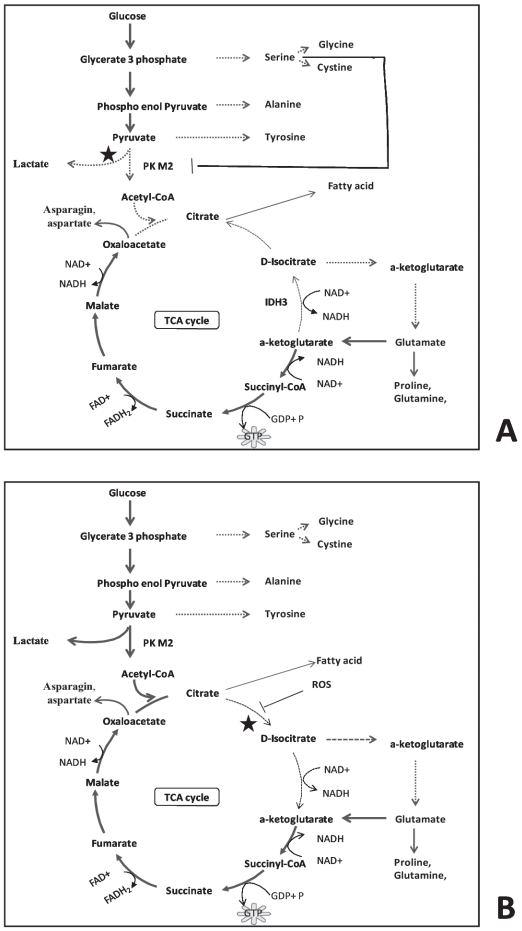
Rank	P-value	Fold Change	Gene
513	3.53E-75	1.52	NDUFC1
918	1.95E-62	1.40	NDUFS3
1469	1.16E-50	1.22	NDUFV3
1611	3.27E-48	1.27	NDUFC2
1623	4.94E-48	1.44	NDUFS8
1753	9.63E-46	1.36	NDUFB2
2456	1.62E-36	1.33	NDUFV2
2888	9.72E-32	1.35	NDUFS2
5720	1.75E-12	1.20	NDUFB3
5932	1.32E-11	1.07	NDUFB4
7094	1.03E-7	1.08	NDUFS1
7534	1.54E-6	1.09	NDUFS5
8372	1.53E-4	1.07	NDUFB6
8556	3.28E-4	1.07	NDUFS7
8812	9.59E-4	1.06	NDUFB5
11664	0.475	1.00	NDUFAF1
11912	0.572	-1.00	NDUFB8
16764	1.000	-1.06	NDUFS6
18257	1.000	-1.17	NDUFV1
18673	1.000	-1.41	NDUFS4



Rank	P-value	Fold Change	Gene
1473	1.28E-50	1.54	SDHC
5627	6.60E-13	1.16	SDHB
13283	0.950	-1.04	SDHA



Isocitrate Dehydrogenase : Isocitrate dehydrogenase (IDH) is another enzyme of TCA cycle that has potential to play key role in remodeling TCA cycle in cancerous cells. It is a reversible enzyme that catalyzes the $NADP^+$-dependent oxidative decarboxylation of isocitrate (ICT) to alpha-ketoglutarate and the NADPH/CO_2-dependent reductive carboxylation of alpha-ketoglutarate to ICT. In World Health Organization grades II–IV gliomas, frequent (~70-80%) mutations have been reported in NADP+-dependent isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2) (37). IDH1 and IDH2 with cancer-associated mutations at the active site are unable to carry out the reductive carboxylation of alpha-ketoglutarate (38-40). These mutants are also defective in ICT decarboxylation and converted alpha-ketoglutarate to 2-hydroxyglutarate using NADPH (38, 39). However, such mutations in IDH3 are not generally present, even in grade II-III gliomas and secondary glioblastomas where IDH1 and IDH2 mutations are frequent (41). These mutations in cytosolic IDH1 have probable advantage in blocking synthesis of glutamine, which helps cancerous cell from overcoming the problem of glutamine synthesis and utilization loop. One possible reason why mutations are absent in mitochondrial IDH3, while they are so frequent in IDH1 and IDH2 is that, these



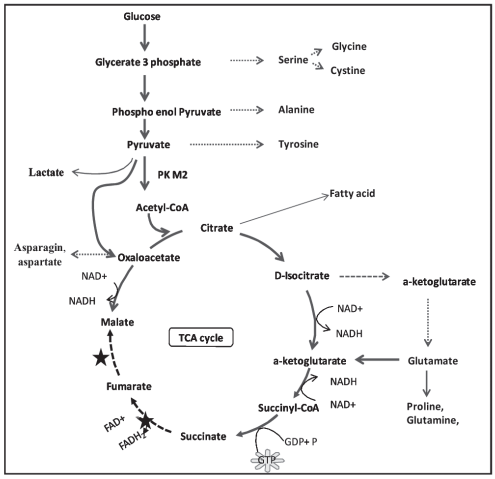


Fig. 1. Truncation of TCA cycle by inhibition/mutation of different enzymes A. Inhibition of PKM2 in absence of serine leads to accumulation of glycolytic intermediates, while TCA cycle components are predominantly synthesized from glutamine carbon, B. Inhibition of aconitase by ROS leads to truncation of TCA cycle, leading to aerobic glycolysis with completion of TCA cycle through anaplerotic conversion of glutamate to alpha ketoglutarate and C. Inhibition of succinate dehydrogenase and fumarate hydratase further leads to truncation of TCA cycle that can be completed through another anaplerotic pyruvate to oxaloacetate conversion.

mutations abolish the carboxylic synthesis of isocitrate from alpha- ketoglutarate. Thereby in a situation when enough glucose carbon is not available for citrate mediated synthesis of required amino acids, IDH3 can convert glutamate to citrate and contribute in biosynthesis of required compounds. Hence, any mutation in IDH3 can be lethal to cancerous cell and not get fixed in progressive cancer.

Succinate dehydrogenase **:** Succinate dehydrogenase (SDH) and fumarate hydratase (FH) are other enzymes that link TCA cycle to nitrogen metabolism and their dysfunction has been implicated in tumorigenesis. Moreover, accumulated succinate and fumarate in response to SDH and FH dysfunction get secreted to cytosol and there they inhibit a family of prolyl hydroxylase enzymes that possibly play a role in tumor maintenance by making the affected cells resistant to certain apoptotic signals (42). However, truncation of TCA cycle at this step can prevent synthesis of oxaloacetate that is essential for synthesis of aspartate. Providentially, another anaplerotic reaction can generate oxaloacetate from pyruvate (Figure 1C). These observations clearly indicate that although almost all steps of TCA cycle are completed but key enzymes are either down regulated or get mutated (possible in advance stage of cancer) to modify the TCA

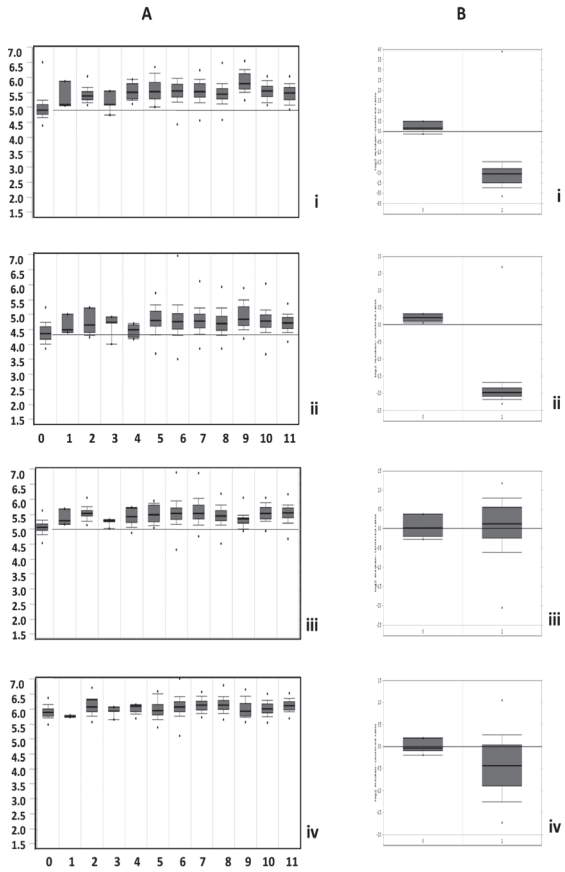


Fig. 2. Expression of four ATP synthase subunits (i.ATP5J2, ii. ATP5H, iii. ATP5G1 and iv. ATP5I) in different breast cancer and stromal tissues A. in normal tissue and 11 different breast cancer types. 0. normal (144) 1. Benign Breast Neoplasm (3) 2. Breast Carcinoma (14) 3. Breast Phyllodes Tumor (5) 4. Ductal Breast Carcinoma in Situ (10) 5. Invasive Breast Carcinoma (21) 6. Invasive Ductal Breast Carcinoma (1,556) 7. Invasive Ductal and Invasive Lobular Breast Carcinoma (90) 8. Invasive Lobular Breast Carcinoma (148) 9. Medullary Breast Carcinoma (32) 10. Mucinous Breast Carcinoma (46) 11. Tubular Breast Carcinoma (67). Numbers in the parenthesis indicates number of samples analyzed. All the different breast cancer types show an increase in expression of ATP synthase subunits. B 1. Normal and 2. Stromal tissue.

cycle for accumulation of intermediates required for synthesis of different amino acids, nucleic and fatty acids. The remaining steps of truncated TCA cycle further get completed using alternate carbon source from the surrounding stromal cells. Intriguingly, in diabetic neuropathy nerve cells also show truncation of TCA cycle but are not able to sustain themselves as they lack glutamine from surroundings to fuel their energy demand unlike cancerous cells that have a surrounding stromal tissue to fuel TCA cycle through glutamate and other metabolites (16, 35). Thus, absolute dependence of cancerous cell on glutamate is for efficient energy production and macromolecule synthesis through multiple carbon sources. Moreover, it has been illustrated in case of HeLa cells that glutamate generates energy as well as macromolecules by aerobic oxidation (10).

Structural adaptations in cancerous cell mitochondria for sustaining oxidative phosphorylation : Structural changes in mitochondria of various cancer cell types have been observed e.g., an increase in mitochondrial mass has been shown (13). Further elongation of mitochondria has been implicated in adaptations to nutrient scarcity and escaping autophagy, in different diseases and growth conditions (43). It seems that these mitochondrial structural changes are made to maximize the utilization of available truncated TCA components for growth of cancerous cells. An alteration in mitochondrial membrane may be to allow insertion of more number of ATP synthase in its membrane. This will not only be an efficient way of energy generation but also explain the consumption of oxygen by cancerous cells. However, this leads to the question whether the electron transport chain is intact and functional in cancerous cell mitochondria or not? Vital mitochondrial assays show that ETC enzymes are not only functional but have increased activity (13). Analysis of Oncomine database (44), further establishes increase in expression of various ETC enzyme coding genes (Figure 2). Here, most notable is that almost all genes of mitochondrial ATP synthase are up regulated in

cancer cell (45). However, in stromal tissue most of the genes coding for ATP synthase subunits are down regulated which are an indication of nonfunctional OXPHOS in stromal cells. Hence, in the light of facts that cancerous cells can synthesize NADH and have high levels of functional ATP synthase enzymes compared to normal cells, it is irresistible to postulate that cancerous cells utilize the efficient way of ATP synthesis (OXPHOS) in addition to the aerobic glycolysis.

Concluding remarks : Altered mitochondrial metabolism in cancerous cells has acquired profuse interest among oncologists worldwide; primarily to target the up-regulated pathways for the development of anticancer drugs. Among these, PKM2, glutamine metabolism and transport are being pursued to design and test specific inhibitors. It is evident that inhibition of any single enzyme cannot result in collapse of complete pathway as subsequent steps get completed through alternative carbon source/s. Further, various metabolic rearrangements seem to fulfill the requirements of continuously dividing cancer cells rather than a cause of cancer. Hence, a cancerous cell already using multiple carbon sources and a number of interlinked pathways at its disposal, may further rearrange its mitochondrial metabolic pathway targeted through a drug. In this background, it is necessary to understand the site of truncation of TCA cycle and its complementary available pathways as simultaneous targeting of these would be an effective therapeutic approach. Further, the implication of breakdown of cancerous cell fueling from surrounding microenvironment in development of effective therapeutic interventions cannot be ignored.

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References

1. Vander Heiden, M.G., Cantley, L.C. and Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation, Science, 324: 1029-1033.
2. Boellaard, R., O'Doherty, M., Weber, W., Mottaghy, F., Lonsdale, M., Stroobants, S., Oyen, W.G., Kotzerke, J., Hoekstra, O., Pruim, J., Marsden, P., Tatsch, K., Hoekstra, C., Visser, E., Arends, B., Verzijlbergen, F., Zijlstra, J., Comans, E.I., Lammertsma, A., Paans, A., Willemsen, A., Beyer, T., Bockisch, A., Schaefer-Prokop, C., Delbeke, D., Baum, R., Chiti, A. and Krause, B. (2010). FDG PET and PET/CT: EANM procedure guidelines for tumour PET imaging: version 1.0, Eur. J. Nucl. Med. Mol. Imaging, 37: 181-200.
3. Gambhir, S.S. (2002). Molecular imaging of cancer with positron emission tomography, Nat Rev Cancer, 2: 683-693.
4. Warburg, O. (1924). Über den Stoffwechsel der Carcinomzelle., Naturwissenschaften, 12: 1132-1137.
5. Warburg, O. (1956). On respiratory impairment in cancer cells, Science, 124: 269-270.
6. Koppenol, W.H., Bounds, P.L. and Dang, C.V. (2011). Otto Warburg's contributions to current concepts of cancer metabolism, Nat Rev Cancer, 11: 325-337.
7. Ayyasamy, V., Owens, K.M., Desouki, M.M., Liang, P., Bakin, A., Thangaraj, K., Buchsbaum, D.J., LoBuglio, A.F. and Singh, K.K. (2011). Cellular Model of Warburg Effect Identifies Tumor Promoting Function of UCP2 in Breast Cancer and Its Suppression by Genipin, PLoS ONE, 6: e24792.
8. Vaishnavi, S.N., Vlassenko, A.G., Rundle, M.M., Snyder, A.Z., Mintun, M.A. and Raichle, M.E. (2010). Regional aerobic glycolysis in the human brain, Proc Natl Acad Sci USA, 107: 17757-17762.
9. Paul, R.J., Bauer, M. and Pease, W. (1979). Vascular smooth muscle: aerobic

glycolysis linked to sodium and potassium transport processes, Science, 206: 1414-1416.

10. Reitzer, L.J., Wice, B.M. and Kennell, D. (1979). Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells, J Biol Chem, 254: 2669-2676.

11. Fernandes, L.C., Marques-da-Costa, M.M. and Curi, R. (1994). Metabolism of glucose, glutamine and pyruvate in lymphocytes from Walker 256 tumor-bearing rats, Braz J Med Biol Res, 27: 2539-2543.

12. Shlomi, T., Benyamini, T., Gottlieb, E., Sharan, R. and Ruppin, E. (2011). Genome-Scale Metabolic Modeling Elucidates the Role of Proliferative Adaptation in Causing the Warburg Effect, PLoS Comput Biol, 7: e1002018.

13. Sotgia, F., Whitaker-Menezes, D., Martinez-Outschoorn, U.E., Flomenberg, N., Birbe, R.C., Witkiewicz, A.K., Howell, A., Philp, N.J., Pestell, R.G. and Lisanti, M.P. (2012). Mitochondrial metabolism in cancer metastasis: visualizing tumor cell mitochondria and the "reverse Warburg effect" in positive lymph node tissue, Cell Cycle, 11: 1445-1454.

14. Martinez-Outschoorn, U.E., Lin, Z., Trimmer, C., Flomenberg, N., Wang, C., Pavlides, S., Pestell, R.G., Howell, A., Sotgia, F. and Lisanti, M.P. (2011). Cancer cells metabolically "fertilize" the tumor microenvironment with hydrogen peroxide, driving the Warburg effect: implications for PET imaging of human tumors, Cell Cycle, 10: 2504-2520.

15. Whitaker-Menezes, D., Martinez-Outschoorn, U.E., Lin, Z., Ertel, A., Flomenberg, N., Witkiewicz, A.K., Birbe, R.C., Howell, A., Pavlides, S., Gandara, R., Pestell, R.G., Sotgia, F., Philp, N.J. and Lisanti, M.P. (2011). Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts, Cell Cycle, 10: 1772-1783.

16. Martinez-Outschoorn, U.E., Pavlides, S., Howell, A., Pestell, R.G., Tanowitz, H.B., Sotgia, F. and Lisanti, M.P. (2011). Stromal-epithelial metabolic coupling in cancer: integrating autophagy and metabolism in the tumor microenvironment, Int J Biochem Cell Biol, 43: 1045-1051.

17. Pavlides, S., Tsirigos, A., Vera, I., Flomenberg, N., Frank, P.G., Casimiro, M.C., Wang, C., Pestell, R.G., Martinez-Outschoorn, U.E., Howell, A., Sotgia, F. and Lisanti, M.P. (2010). Transcriptional evidence for the "Reverse Warburg Effect" in human breast cancer tumor stroma and metastasis: similarities with oxidative stress, inflammation, Alzheimer's disease, and "Neuron-Glia Metabolic Coupling", Aging (Albany NY), 2: 185-199.

18. DeBerardinis, R.J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S. and Thompson, C.B. (2007). Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis, Proc Natl Acad Sci U S A, 104: 19345-19350.

19. Desideri, E., Vegliante, R. and Ciriolo, M.R. (2014). Mitochondrial dysfunctions in cancer: Genetic defects and oncogenic signaling impinging on TCA cycle activity, Cancer letters, PMID: 24614286.

20. Zheng, J. (2012). Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review), Oncol Lett, 4: 1151-1157.

21. Shuch, B., Linehan, W.M. and Srinivasan, R. (2013). Aerobic glycolysis: a novel target in kidney cancer, Expert Rev Anticancer Ther, 13: 711-719.

22. Singleterry, J., Sreedhar, A. and Zhao, Y. (2014). Components of cancer metabolism

and therapeutic interventions, Mitochondrion, 17: 50-55.

23. Filipp, F.V., Ratnikov, B., De Ingeniis, J., Smith, J.W., Osterman, A.L. and Scott, D.A. (2012). Glutamine-fueled mitochondrial metabolism is decoupled from glycolysis in melanoma, Pigment Cell Melanoma Res, 25: 732-739.

24. Icard, P. and Lincet, H. (2012). A global view of the biochemical pathways involved in the regulation of the metabolism of cancer cells, Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1826: 423-433.

25. Raimundo, N., Baysal, B.E. and Shadel, G.S. (2011). Revisiting the TCA cycle: signaling to tumor formation, Trends Mol Med, 17: 641-649.

26. Wallace, D.C. (2012). Mitochondria and cancer, Nat Rev Cancer, 12: 685-698.

27. Lehninger, A., Nelson, D.L. and Cox, M.M. (2008). Lehninger Principles of Biochemistry, W.H. Freeman & Co., New York, USA.

28. Icard, P., Poulain, L. and Lincet, H. (2012). Understanding the central role of citrate in the metabolism of cancer cells, Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1825: 111-116.

29. Wong, N., De Melo, J. and Tang, D. (2013). PKM2, a Central Point of Regulation in Cancer Metabolism, Int J Biochem Cell Biol, 2013: 11.

30. Christofk, H.R., Vander Heiden, M.G., Harris, M.H., Ramanathan, A., Gerszten, R.E., Wei, R., Fleming, M.D., Schreiber, S.L. and Cantley, L.C. (2008). The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth, Nature, 452: 230-233.

31. Chaneton, B., Hillmann, P., Zheng, L., Martin, A.C.L., Maddocks, O.D.K., Chokkathukalam, A., Coyle, J.E., Jankevics, A., Holding, F.P., Vousden, K.H., Frezza, C., O/'Reilly, M. and Gottlieb, E. (2012). Serine is a natural ligand and allosteric activator of pyruvate kinase M2, Nature, 491: 458-462.

32. Wise, D.R., Ward, P.S., Shay, J.E.S., Cross, J.R., Gruber, J.J., Sachdeva, U.M., Platt, J.M., DeMatteo, R.G., Simon, M.C. and Thompson, C.B. (2011). Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of a-ketoglutarate to citrate to support cell growth and viability, Proc Natl Acad Sci, 108: 19611-19616.

33. Gardner, P.R., Raineri, I., Epstein, L.B. and White, C.W. (1995). Superoxide radical and iron modulate aconitase activity in mammalian cells, J Biol Chem, 270: 13399-13405.

34. Waris, G. and Ahsan, H. (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions, J Carcinog, 5: 14.

35. Hinder, L.M., Vincent, A.M., Burant, C.F., Pennathur, S. and Feldman, E.L. (2012). Bioenergetics in diabetic neuropathy: what we need to know, J Peripher Nerv Syst, 17: 10-14.

36. Gupta, K.J., Shah, J.K., Brotman, Y., Jahnke, K., Willmitzer, L., Kaiser, W.M., Bauwe, H. and Igamberdiev, A.U. (2012). Inhibition of aconitase by nitric oxide leads to induction of the alternative oxidase and to a shift of metabolism towards biosynthesis of amino acids, J Exp Bot, 63: 1773-1784.

37. Yen, K.E. and Schenkein, D.P. (2012). Cancer- associated isocitrate dehydrogenase mutations, Oncologist, 17: 5-8.

38. Leonardi, R., Subramanian, C., Jackowski, S. and Rock, C.O. (2012). Cancer-associated Isocitrate Dehydrogenase Mutations Inactivate NADPH-dependent Reductive Carboxylation, J Biol Chem, 287: 14615-14620.

39. Kloosterhof, N.K., Bralten, L.B., Dubbink, H.J., French, P.J. and van den Bent, M.J. (2011). Isocitrate dehydrogenase-1 mutations: a fundamentally new understanding of diffuse glioma?, Lancet Oncol, 12: 83-91.

40. Reitman, Z.J. and Yan, H. (2012). Isocitrate Dehydrogenase 1 and 2 Mutations in Cancer: Alterations at a Crossroads of Cellular Metabolism, J Natl Cancer Inst, 102: 932-941.

41. Krell, D., Assoku, M., Galloway, M., Mulholland, P., Tomlinson, I. and Bardella, C. (2011). Screen for IDH1, IDH2, IDH3, D2HGDH and L2HGDH mutations in glioblastoma, PLoS One, 6: e19868.

42. King, A., M.A., S. and Gottlieb, E. (2006). Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer, Oncogene, 25: 4675-4682.

43. Gomes, L.C., Benedetto, G.D. and Scorrano, L. (2011). During autophagy mitochondria elongate, are spared from degradation and sustain cell viability, Nat Cell Biol, 13: 589-598.

44. Rhodes, D.R., Kalyana-Sundaram, S., Mahavisno, V., Varambally, R., Yu, J., Briggs, B.B., Barrette, T.R., Anstet, M.J., Kincead-Beal, C., Kulkarni, P., Varambally, S., Ghosh, D. and Chinnaiyan, A.M. (2007). Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles, Neoplasia, 9: 166-180.

45. Curtis, C., Shah, S.P., Chin, S.F., Turashvili, G., Rueda, O.M., Dunning, M.J., Speed, D., Lynch, A.G., Samarajiwa, S., Yuan, Y., Graf, S., Ha, G., Haffari, G., Bashashati, A., Russell, R., McKinney, S., Langerod, A., Green, A., Provenzano, E., Wishart, G., Pinder, S., Watson, P., Markowetz, F., Murphy, L., Ellis, I., Purushotham, A., Borresen-Dale, A.L., Brenton, J.D., Tavare, S., Caldas, C. and Aparicio, S. (2012). The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups, Nature, 486: 346-352.

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Nutraceuticals from Marine Derived Krill Oil with Immense Heath Potentials

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Abstract

Krill oil derived from miniature marine crustaceans has been discovered to be opulent with health promoting components. The oil is rich source of phospholipids, ω-3 -fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DPA), also the antioxidant astaxanthin. Krill oil is now considered to be superior to the customary fish oil owing to its stability, less mercury and better bioavailability of the fatty acids. It is known to confer numerous health benefits such as cholesterol lowering, cardio protection, antiarthritic effect, suppression of hepatic steatosis, cancer remediation, menstrual disorder alleviation and cognitive enrichment. This review explores the validated and claimed health benefits of krill oil. Scouring the literature database revealed various stages of krill oil research providing fertile ground to further explore.

Key words: Krill oil, dietary supplement, omega-3 fatty acids, astaxanthin, cardioprotective.

Introduction

Krills are pelagic, marine shrimp-like crustaceans from the class Malacostraca. They are tiny (generally, 1-2 cm or maximum 6-15 cm) and exist in huge swarms and feed on phytoplanktons (Fig 1A and 1B) (1). They are found in enormous groups particularly in Antarctica. They form an essential link in the marine food chain, serving as food for whale, manta ray, seals, squid, penguins, cormorants and other sea birds (2). In fact, in Norwegian language 'krill' means 'whale food'. There are about 85 species, out of which Antarctic krill (*Euphausia superba*) is the most abundant species, a keystone food web species (3). Krill has attracted attention of nutraceutical industry for abundance of bioactive compounds such as ω-3 fatty acids, choline and a carotenoid antioxidant astaxanthin. Both components of ω-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential for metabolism. Their deficiency causes fatigue, memory decline, dry skin, heart problems, mood swings or depression, poor endothelial function and obstruction of microcirculation (4). Fish oil has been the traditional supplement for augmenting ω-3 fatty acids in the human diet. Recently krill oil has emerged as more potent source of this essential fatty acid. Antarctic krill (*Euphausia superba*) oil is considered to be very therapeutic. The oil has been proven to alleviate inflammation, reduce cholesterol, boost cardiac, hepatic and renal health, protect from cancer, relieve premenstrual syndrome, reduce arthritis pain and improve brain function (5, 6, 7). Major brands of krill oils are Neptune, Schiff Megared, Jarrow Formulas, Dr. Mercola, Twinlab, Swanson and Vitacost (7). Krill protein concentrate contains high-quality protein (77.7% dry mass basis) and lipids (8.1% dry mass basis) (8).

Nutritional profile : A high performance liquid chromatography-electrospray tandem mass spectrometry was employed to elucidate the phospholipids in krill oil (9). A total of 69 choline-containing phospholipids were detected and the

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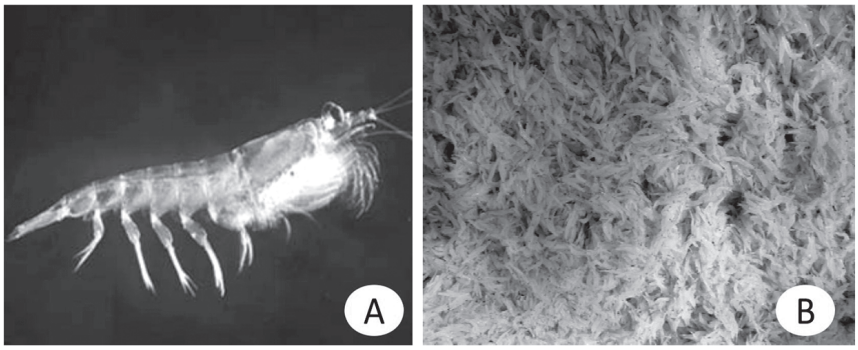

Fig. 1. (A) Single krill (the red-orange pigments are astaxanthin) (B) Freshly harvested krills

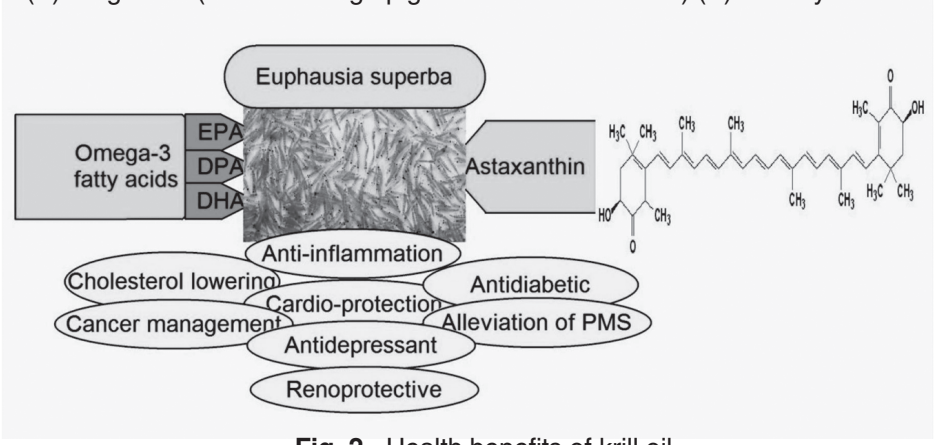

Fig. 2. Health benefits of krill oil

phosphatidylcholine concentration was estimated to be 34 g/100 g oil. These results confirm the complexity of the phospholipid composition of krill oil and the presence of long chained, heavily unsaturated fatty acids. Astaxanthins are marine carotenoid pigments with strong antioxidant, anti-inflammatory, anti-cancer, anti-obesity and insulin-sensitivity potential. The mechanisms underlying the insulin sensitizing effects of astaxanthin, derived from marine algae was investiagted in high fat-high fructose diet fed insulin-resistant mice (10). Astaxanthin supplementation led to normalization of increased body weight, hyperglycemia, hyperinsulinemia, hyperlipidemia and increased plasma levels of tumor necrosis factor-α (TNF-α) and interleukin-6. It was demonstrated that long-term astaxanthin administration improves insulin sensitivity by activating the post-receptor insulin signaling and by reducing oxidative stress, lipid accumulation and proinflammatory cytokines in obese mice. Unlike algal astaxanthins, krill oil-derived astaxanthin has not been explored much. However, the above findings build prospect for similar outcome.

Health benefits of krill oil : Many health promoting aspects of krill has been validated so far. Fig 2 depicts the applications.

Anti-inflammation : The effect of Neptune krill oil on C-reactive protein (CRP) was evaluated in patients with chronic inflammation, especially on arthritic symptoms through a randomized, double blind, placebo controlled the study involving 90 patients (6). After 7 days of treatment, the oil reduced CRP by 19.3% as compared to an

increase by 15.7% with placebo. After 14 and 30 days of treatment, the oil further decreased CRP by 29.7% and 30.9% respectively. After 7 days of treatment, the krill oil reduced pain scores by 28.9%, reduced stiffness by 20.3% and reduced functional impairment by 22.8%.The results of study indicated that the krill oil at a daily dose of 300 mg significantly inhibits inflammation and reduces the arthritic symptoms within a short treatment period of 7 and 14 days (6). The effects of dietary ω-*3* long chain polyunsaturated fatty acids (ω-3 LCPUFA) in the form of fish oil and krill oil were compared. In rats fed with the PUFA diet for 4 week, liver triglycerides and the peritoneal macrophage response to an inflammatory stimulus were significantly lower than those fed the control diet. Heart triglycerides were lower only in krill oil-fed rats. These effects were associated with a lower concentration of the endocannabinoids, anandamide and 2-arachidonoylglycerol, in the visceral adipose tissue and of anandamide in the liver and heart, which, in turn, was associated with lower levels of arachidonic acid in membrane phospholipids, but not with higher activity of endocannabinoid-degrading enzymes. The reduction of substrates for inflammatory molecules and endocannabinoids may account for the dampened inflammatory response and the physiological re-equilibration of body fat deposition in obese rats (11). A standardised preparation of krill oil and fish oil was evaluated in mice model for arthritis. The level of EPA + DHA was 0.44 g/100 g in the krill oil diet and 0.47 g/100 g in the fish oil diet. The consumption of krill oil and fish oil-supplemented diet significantly reduced the arthritis scores and hind paw swelling when compared to a control diet not supplemented with EPA and DHA. However, the arthritis score during the late phase of the study was only significantly reduced after krill oil administration. Furthermore, mice fed the krill oil diet demonstrated lower infiltration of inflammatory cells into the joint and synovial layer hyperplasia as compared to the control (12). The effects of krill oil on inflammation and redox status were evaluated in dextran sulfate sodium (DSS)-induced colitis in rats (13). The colon length was significantly preserved after krill oil diet. Prostaglandin (PGE3) increased significantly in the krill oil group. Peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1α (Pparg1α) expression increased and the levels of protein oxidation markers decreased significantly in this group. Based on the elicited effects, it was inferred that krill oil exerts protection against DSS induced-colitis (13).

Cholesterol lowering and anti-obesity effect : Krill oil has shown efficacy for the management of hyperlipidemia. A 3-month randomized study was conducted to assess the effects of this oil on blood lipids, specifically total cholesterol, triglycerides, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) of 120 patients with hyperlipidemia (14). Krill oil taken at a dose of 1-3 g per day body mass index (BMI) was found to be effective for the reduction of glucose, total cholesterol, triglycerides, LDL and HDL, as compared to both fish oil and placebo (14). A randomized, double-blind study was conducted on 76 volunteers to determine the role of krill oil. When capsules containing 2 g/d of krill oil was administered for 4 weeks, plasma EPA and DHA concentrations increased significantly while blood urea nitrogen declined (15). The digestibility, tissue deposition, metabolism and tissue oxidative stability of the ω-3 PUFA were determined (8). Rats were fed *ad libitum* (as much as desired) isocaloric diets for 4 weeks with either 10% freeze-dried krill protein isolate or 10% casein, both added in corn oil. Fatty acid compositions of various tissues were analyzed by gas chromatography. Lipid peroxidation was determined by thiobarbituric acid reactive substances (TBARS). Total antioxidant capacity and urinary eicosanoid metabolites were determined by enzyme immunoassay. DHA concentration in the brain increased, while both DHA and EPA content in fat pads and liver increased. The ω-6 fatty acid, arachidonic acid decreased. Feeding the krill protein concentrate diet decreased pro-inflammatory 2-series prostaglandin and thromboxane metabolites (8). It was investigated whether an intake of 2 g/d of

krill oil is able to modulate the level of plasma endocannabinoids in overweight and obese subjects. Increased levels of endocannabinoids were reported in overweight and obese subject with respect to normo-weight subjects. Krill oil was able to significantly decrease the endocannabinoid 2-arachidonoylglycerol (2-AG) selectively in obese subjects (16). The efficacy of two different sources of ω-3 PUFAs were evaluated on the liver of mice fed diets (17). The supplement derived from a phospholipid krill fraction downregulated the activity of pathways involved in hepatic glucose production as well as lipid and cholesterol synthesis. The data also suggested that the krill oil-supplementation increases the activity of the mitochondrial respiratory chain. Same dose of EPA and DHA derived from fish oil modulated fewer pathways than that of krill oil. Also, it did not modulate key metabolic pathways influenced by krill oil *viz*. glucose metabolism, lipid metabolism and the mitochondrial respiratory chain. Moreover, fish oil upregulated the cholesterol synthesis pathway, whereas krill oil showed the reverse effect (17). The influence of both oils on lipid homeostasis and inflammation was investigated in mice with persistent low-grade exposure to human TNF-α. Further, the roles of the structural forms of EPA and DHA were explored. A 6 week feeding with krill oil could modulae lipid metabolism by lowering plasma levels of triglycerides and cholesterol, and stimulating the mitochondrial and peroxisomal fatty acid β-oxidation, as well as improving the overall carnitine turnover. When quantitatively similar doses of ω-3 PUFAs are administered, krill oil seems to have a greater potential to promote lipid catabolism (18). A double-blind, crossover trial was performed on 12 persons to compare the the uptake of EPA+DHA formulations derived from fish oil and krill oil (19). When capsules containing 1680 mg EPA+DHA were given, the highest incorporation into plasma phospholipd was by krill oil. Fatty acid analysis of the supplements showed that the krill oil contained 22% of the total EPA amount in free form, and 21% of the total DHA as free DHA, while the fish oil did not contain any free fatty acid (19). The effects of krill oil (whole as well as phospholipid-type krill oil) on plasma cholesterol and glucose levels were investiagated in high-cholesterol diet-fed rats (20). The whole krill oil contained 37.63% triglycerides, 48.37% phospholipids, 13.54% free fatty acids and 0.66% cholesterol; whereas, for the phospholipid type, these parameters measured 0.59, 69.80, 28.53 and 1.09%, respectively. The phospholipid-type krill oil contained more PUFA (37.76%) than whole krill oil (28.36%). The intake of both forms of krill oil for 4 weeks caused a significant reduction in body weight gain, plasma levels of total cholesterol and LDL cholesterol in high carbohydrate diet-fed rats. Phospholipid-type krill oil was more effective in decreasing the above two parameters in plasma, which was credited to the higher ω-3 PUFA levels (20).

Cardioprotection : The effects of krill oil on serum lipids of hyperlipidemic rats were evaluated. Total cholesterol and LDL showed significant decrease, building promise that its consumption may be healthful (21). The dose-dependent effects of dietary ω-3 PUFA supplementation given as krill oil, was investigated on metabolic parameters in high fat diet-fed mice (22). Eight-week high fat diet increased endocannabinoid levels in most of the body tissues. Krill oil reduced anandamide and/or 2-arachidonoylglycerol levels in all tissues except the liver, in a dose-dependent manner. The levels of endocannabinoid precursors were down-regulated, indicating that krill oil affects levels of endocannabinoids in part by reducing the availability of their biosynthetic precursors (22). The effects of krill oil on cardiac remodeling after experimental myocardial infarction was investigated in rat models. The animals were pretreated with krill oil, 2 weeks prior to induction of the infarction. After 7 days of infarction, the rats were examined with echocardiography. The evaluation showed significant attenuation of left ventricular dilatation in the group pretreated with krill oil as compared to the control. Lowered heart weight, lung weight, and levels of mRNA encoding classical markers of left ventricular stress, matrix

remodeling and inflammation was observed. It became clear that supplementation with krill oil leads to a proportional increase of n-3 PUFA in myocardial tissue and its administration before infarction induction, attenuates left ventricular remodelling (23).

Alleviation of hepatic steatosis : The effects of dietary krill oil on cardiometabolic risk factors were investigated in mice fed with a high-fat diet for 8 weeks (24). The krill oil supplementation (1.25, 2.5 and 5 wt%) caused a significant reduction in liver weight (i.e., hepatomegaly) and total liver fat (i.e., hepatic steatosis), due to a dose-dependent reduction in hepatic triglyceride and cholesterol. Serum cholesterol levels and blood glucose was reduced. Serum adiponectin was increased in krill oil-fed animals. It was confirmed that dietary krill oil is effective in improving metabolic parameters in mice fed a high-fat diet and beneficial for patients with the metabolic syndrome and/or nonalcoholic fatty liver disease (24). A time-dependent decrease in the activities of the mitochondrial tricarboxylate carrier and lipogenic enzymes was reported in rats fed with a diet enriched with 2.5% krill oil. It induced inhibition of hepatic lipogenesis. The decrease in the activity of the mitochondrial tricarboxylate carrier was traced to the reduced expression of the protein. Greater reduction in the levels of hepatic triglycerides and cholesterol was found in comparison to fish oil-fed rats (25). Also, it was reported that hepatic triglyceride and cholesterol accumulation is accompanied by the reduction in plasma levels of triglycerides and glucose; also by the prevention of a plasma insulin increase. A significant increase in the activity of carnitine palmitoyl-transferase I and the levels of carnitine was also observed, suggesting a concomitant stimulation of hepatic fatty acid oxidation (26).

Antiglycemic effect : The effect of krill oil on glucose tolerance in obese rabbits was studied (27). Results showed that the supplementation decreases fasting blood glucose and improves glucose tolerance in the test animals. Induction of insulin sensitivity and insulin secretion and modified gene expressions of some key enzymes involved in β-oxidation and lipogenesis in liver and skeletal muscle was assumed to the mechanism.

Cancer management : Krill oil is claimed to be efficient in inhibiting many types of cancer *viz*. colon, breast and skin. The effects of krill oil on human colon cancer cells SW480 was evaluated by (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) MTT method (21). A time-dependent inhibition of cell growth was observed that suggested cancer prophylaxis potential of krill oil. The possible inhibitory effect of astaxanthin against inflammation-related mouse colon carcinogenesis and dextran sulfate sodium (DSS)-induced colitis was investigated in mice. Its intake led to significant inhibition in the occurrence of colonic mucosal ulcers, dysplastic crypts and colonic adenocarcinoma at week 20. Astaxanthin-feeding suppressed the expression of inflammatory cytokines, including nuclear factor (NF)-kB, (TNF)-α and interleukin (IL)-1β; also inhibited proliferation, and induced apoptosis in the colonic adenocarcinomas. When fed at 200 ppm dose, it significantly inhibited the development of induced colitis. It also lowered the protein expression of NF-kB, and the mRNA expression of inflammatory cytokines, including IL-1β, IL-6, and cyclooxygenase (COX)-2 (28). The results suggested that the dietary astaxanthin suppresses colitis and its-related colon carcinogenesis, partly through inhibition of the expression of inflammatory cytokine and proliferation. However, further research inputs are warranted for more concrete findings (28).

Suppression of PMS symptoms

The symptoms premenstrual syndrome (PMS) are cranky feeling, bloating and mood swings, which sometimes assume severe form. The effectiveness of Neptune krill oil (NKO) in the management of PMS and dysmenorrhea was evaluated and compared with that of fish oil (7). A double-blind, randomized clinical trial involving 70 PMS patients was conducted. After a treatment period of three months with both the oils, self-

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assessment questionnaire was administered. The data analysis clearly indicated reduction in the number of analgesics used for dysmenorrhea in the krill oil group. It was inferred that NKO can significantly assuage dysmenorrhea and the emotional symptoms of PMS. Richness of krill oil in ω–3 fatty acid is regarded responsible for the beneficial effect. Better balance of hormonal and chemical changes is believed to suppress the unpleasant symptoms and provide relief (7).

Cognitive and antidepressant effect : Both DHA and EPA generate neuroprotective metabolites that benefit attention deficit/ hyperactivity disorder, autism, dyspraxia, dyslexia and aggression (29). Promising results in schizophrenia and borderline personality disorder have been observed. The cognitive decline and mild cognitive impairment correlate with lowered tissue levels of DHA/EPA, which showed improvement on their supplementation. Huntington disease (muscle coordination and leads to cognitive deterioration and psychiatric problems) has responded to EPA. The effects of krill oil on cognition and depression-like behaviour were investiagted in rats. After 7 week intake of the oil, the test animals showed better discrimination between the active and the inactive levers in the Aversive Light Stimulus Avoidance Test (ALSAT) from day 1 of training. Krill oil prevented resignation/depression on the third day in the Unavoidable Aversive Light Stimulus Test (UALST) (29). A shorter immobility time was observed for the krill oil and imipramine (standard antidepressant) groups compared to the control in the Forced Swimming Test. The mRNA for brain-derived neurotrophic factor (Bdnf) was specifically upregulated in the hippocampus of female rats receiving 7 weeks of krill oil supplementation. Males also exhibited an increase in prefrontal cortex expression of Arc mRNA, a key protein in long-term synaptic plasticity. It was confirmed that krill oil facilitates learning processes and provides antidepressant-like effects (29). For its cognitive-enhancement and cerebral stimulation of foetus brain, it is recommended for pregnant women.

Renoprotective effect : Nephrocalcinosis, a common renal abnormality has been implicated in subsequent renal failure in rats. The effect of 4 week consumption of krill protein concentrate was evaluated on renal health of rats and compared with that of casein (30). Tissue analyses showed that rats fed the protein concentratae had lower urinary n-acetyl glucosaminidase levels and minimal microtubular calcium deposition compared to rats fed casein. There was a tendency for higher glomerular filtration rates and lower proteinuria, and higher urinary output in rats fed krill protein concentrate compared to casein. Krill protein concentrate is expected to avert early renal injury and thus reduce nephrocalcinosis threat (30).

Extraction : The effect of pressure, temperature and extraction time on krill oil was assessed (31). The maximum oil yield was found at higher extraction temperature and pressure. The oil obtained by supercritical carbon dioxide (SC-CO_2) extraction was more stable, contained a high percentage of EPA and DHA; whereas the acidity and peroxide value of krill oil obtained were lower than that of the oil obtained by hexane (31). The maximum yield of astaxanthin was found in krill oil extracted at 25 MPa and 45°.

Novelty : Omega-3 fatty acids can be obtained from several sources apart from krill oil *viz.* algae, fish oil, plant, enriched dairy products, animal-derived food, seal oil (32). The advantage of phospholipid form of krill oil is that it gets better absorbed and transported as compared to the normal triglyceride form found in fish oil (33). Phospholipids spontaneously form micelles, which can be transported easily in the aqueous environment (25). Also, phospholipids can be absorbed intact or in their lysophosphatidylcholine form. A randomized study was conducted to find out the efficacy of krill oil and fish oil and the better between the two (34). Six capsules of krill oil or three capsules of fish oil were given daily for 7 weeks. EPA and DHA dose in the krill oil was 62.8% of that in the fish oil, yet it showed comparable increase in plasma EPA, DHA and DPA (34). Intake of krill oil does not produce fishy

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burps or belching as this happens in case of fish oil. Krill occupies lower position in food chain, so it is free of heavy metals and other contaminants unlike the latter.

Issues encountered : The exoskeleton of krill contains fluorine, which is toxic in high concentrations, so it must be peeled before consumption. Krill oil is one of the most sensitive oils to rancidity. It requires immediate refrigeration after harvesting for keeping it unoxidized. The increase in demand for ω-3 fatty acid is likely to increase pressure on marine living resources. Overharvesting may result in sharp decline or extinction and may also disrupt the food chain. Further, the impact of emissions (global warming, ozone depletion, acidification and eutrophication), material and energy demands needs assessment. The cost of krill harvesting was assessed and the cost of fossil fuel burnt for operation of fishing vessel, transport and resupply vessel was found high. Concerns have been voiced in recent years regarding the environmental implications of the Antarctic krill fishery (35).

Future directions : Astaxanthin was able to prevent oxidative modification in lymphocytes from rat lymph nodes through the suppression of the oxidative stress condition imposed by fish oil. It was reported that the association of astaxanthin with fish oil could be a good strategy to potentiate immuno-modulatory effects of the latter (36). Many krill-based food products are expected in the near future. While some countries have been consuming it since long, in some other regions, its incorporation in food is at experimental stage. In Japan, it is ingredients in several seafoods. In Norway, krill paste used as dip. Russia is engaged in commercializing krill-based food products. Fermented krill (kapi) is a protein-rich, traditional food in Thai cuisine (Fig 3). Water-soluble fraction of this fermented product possessed radical-scavenging activity, as determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) methods (37). It also showed ferric reducing antioxidant power (FRAP) in a concentration-dependent manner. The fermented product demonstrated high stability to temperature (37). Currently, krill is being consumed as snacks, pizza toppings, omelettes, paste in soups and salads. Raw and boiled krill, krill meal and oil are being marketed for human as well as pet consumption. In recent times, many patents have been filed on novel krill meal cooking, powdering protocols and methods to use krill oil for treatment of metabolic syndromes.

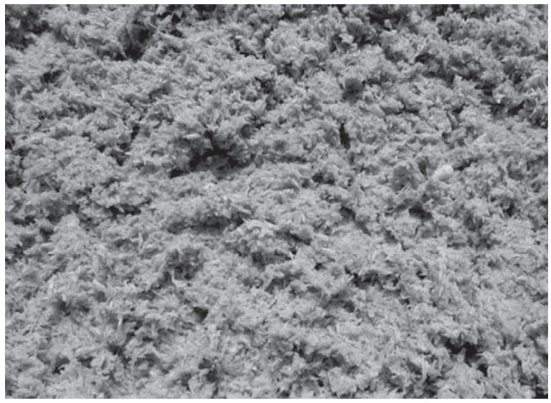

Fig 3. Fermented krills (kapi)

Conclusions

The findings obtained so far are testimony to the krill oil's effective nutritional attributes. More research input can reveal several other clinical applications. In this era of over-exploitation of bio-resources, krill is surprisingly under-utilized. These zooplanktons might tackle the global issue of looming starvation. Krill-based food developments are already picking up in many countries. While taking benefit of the generous marine supply, a balance must be striken not to deplete it for sustainable harvest and protection of ocean health. Krill oil is a relatively nascent field of nutraceutical research, given due emphasis, it could help tackle many health issues.

References

1. Schmidt, K. (2010) Food and feeding in Northern krill (*Meganyctiphanes norvegica* Sars). Advances in Marine Biology, 57: 127-171

2. Trivelpiece, W.Z., Hinke, J.T., Miller, A.K., Reiss, C.S., Trivelpiece, S.G. and Watters, G.M. (2011) Variability in krill biomass links harvesting and climate warming to penguin population changes in Antarctica. Proceedings of the National Academy of Sciences of the United States of America, 108: 7625-7628

3. Reid, K. and Croxall, J.P. (2001) Environmental response of upper trophic-level predators reveals a system change in an Antarctic marine ecosystem. Proceedings of the Royal Society B: Biological Sciences 268: 377-384

4. Vasil'ev A.P., Strel'tsova, N.N. and Sekisova M.A. (2009) Effect of omega-3 fatty acids on the serum lipid profile and microcirculation in patients with metabolic syndrome and hypertensive disease]. Klinicheskaia Meditsina (Moskva), 87: 37-41

5. Berge, K., Musa-Veloso, K., Harwood, M., Hoem, N. and Burri, L. (2014) Krill oil supplementation lowers serum troglycerides without increasing low-density lipoprotein cholesterol in adults with borderline high or high triglyceride levels. Nutrition Research, 34: 126-133

6. Deutsch, L. (2007). Evaluation of the effect of Neptune krill oil on chronic inflammation and arthritic symptoms. Journal of American College of Nutrition, 26: 39-48.

7. Sampalis, F., Bunea R., Pelland M.F., Kowalski, O., Duguet, N. and Dupuis, S. (2003). Evaluation of the effects of Neptune krill oil on the management of premenstrual syndrome and dysmenorrhea. Alternative Medicine Review, 8: 171-179.

8. Bridges, K.M., Giglioti, J.C., Altman, S., Jaczynski, J. and Tou, J.C. (2010). Determination of digestibility, tissue deposition, and metabolism of the omega-3 fatty acid content of krill protein concentrate in growing rats. Journal of *Agricultural* and *Food* Chemistry, 58: 2830-2837.

9. Winther, B., Hoem, N., Berge, K. and Reubsaet, L. (2011). Elucidation of phosphatidylcholine composition in krill oil extracted from *Euphausia superba*. Lipids, 46: 25-36.

10. Arunkumar, E., Bhuvaneswari, S. and Anuradha, C.V. (2012). An intervention study in obese mice with astaxanthin, a marine carotenoid—effects on insulin signaling and pro-inflammatory cytokines. Food and Function, 3: 120-126.

11. Batetta, B., Griinari, M., Carta, G., Murru, E., Ligresti, A., Cordeddu, L., Giordano, E., Sanna, F., Bisogno, T., Uda, S., Collu, M., Bruheim, I., Di Marzo, V. and Banni, S. (2009). Endocannabinoids may mediate the ability of (n-3) fatty acids to reduce ectopic fat and inflammatory mediators in obese Zucker rats. Journal of Nutrition, 139: 1495-1501.

12. Lerna, M., Kerr, A., Scales, H., Berge, K. and Griinari, M. (2010). Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. BMC Musculoskeletal Disorders, 11:136.

13. Grimstad, T., Bjorndal, B., Cacabelos, D., Aasprong, O.G., Janssen, E.A., Omdal, R., Svardal, A., Hausken, T., Bohov, P., Portero-Otin, M., Pamplona, R. and Berge, R.K. (2012). Dietary supplementation of krill oil attenuates inflammation and oxidative stress in experimental ulcerative colitis in rats. Scandinavian Journal of Gastroenterology, 47:49-58.

14. Bunea, R., El Farrah, K. and Deutsch, L. (2004). Evaluation of the effects of Neptune Krill Oil on the clinical course of hyperlipidemia. *Alternative Medicine Review,* 9: 420-428.

15. Maki, K.C., Reeves, M.S., Farmer, M., Griinari, M., Berge, K., Vik, H., Hubacher, R. and Rains, T.M. (2009). Krill oil supplementation increases plasma concentrations of eicosapentaenoic and docosahexaenoic acids in overweight and obese men and women. Nutrition Research, 29: 609-615.

16. Banni, S., Carta, G., Murru, E., Cordeddu, L., Giordano, E., Sirigu, A.R., Berge, K, Vik, H., Maki, K.C., Di Marzo, V. and Griinari, M. (2011). Krill oil significantly decreases 2-arachidonoylglycerol plasma levels in obese subjects. Nutrition and Metabolism (Lond), 8: b7.

17. Burri, L., Berge, K., Wibrand, K., Berge, R.K. and Barger, J.L. (2011). Differential effects of krill oil and fish oil on the hepatic transcriptome in mice. Frontiers in Genetics, 2: b45.

18. Vigerust, N.F., Bjorndal, B., Bohov, P., Brattelid, T., Svardal, A. and Berge, R.K. (2012). Krill oil versus fish oil in modulation of inflammation and lipid metabolism in mice transgenic for TNF-á. European Journal of Nutrition, 52: 1315-1325.

19. Schuchardt, J.P., Schneider, I., Meyer, H., Neubronner, J., von Schacky, C. and Hahn, A. (2011). Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations—a comparative bioavailability study of fish oil vs. Krill oil. Lipids in Health and Disease, 10: 145.

20. Li, D.M., Zhou, D.Y., Zhu, B.W., Chi, Y.L., Sun, L.M., Dong, X.P., Qin, L., Qiao, W.Z. and Murata, Y. (2013). Effects of krill oil intake on plasma cholesterol and glucose levels in rats fed a high-cholesterol diet. *Journal* of the *Science* of *Food* and *Agriculture*, doi: 10.1002/jsfa.6072.

21. Zhu, J.J., Shi, J.H., Qian, W.B., Cai, Z.Z. and Li, D. (2008). Effects of krill oil on serum lipids of hyperlipidemic rats and human SW480 cells. Lipids in Health and Disease, 7: 30.

22. Piscitelli, F., Carta, G., Bisogno, T., Murru, E., Cordeddu, L., Berge, K., Tandy, S., Cohn, J.S., Griinari, M., Banni, S. and Di Marzo, V. (2011). Effect of dietary krill oil supplementation on the endocannabinoidome of metabolically relevant tissues from high-fat-fed mice. Nutrition and Metabolism (Lond), 8: 51.

23. Fosshaug, L.E., Berge, R.K., Beitnes, J.O., Berge, K., Vik, H., Aukrust, P., Gullestad, L., Vinge L.E. and Oie, E. (2011). Krill oil attenuates left ventricular dilate tion after myocardial infarction in rats. Lipids in Health and Disease, 10: 245.

24. Tandy, S, Chung, R.W., Wat, E., Kamili, A., Berge, K., Griinari, M. and Cohn, J.S. (2009). Dietary krill oil supplementation reduces hepatic steatosis, glycemia, and hypercholesterolemia in high-fat-fed mice. Journal of Agricultural and Food Chemistry, 57:9339-9345.

25. Ferramosca, A., Conte, L. and Zara, V. (2012a). A krill oil supplemented diet reduces the activities of the mitochondrial tricarboxylate carrier and of the cytosolic lipogenic enzymes in rats. Journal of Animal Physiology and Animal Nutrition (Berl), 96: 295-306.

26. Ferramosca, A., Conte, A., Burri, L., Berge, K., De Nuccio, F., Giudetti, A.M. and Zara, V. (2012b). A krill oil supplemented diet suppresses hepatic steatosis in high-fat fed rats. PLoS One, 7: e3879.

27. Ivanova, Z., Bjorndal, B., Grigorova, N., Roussenov, A., Vachkova, E., Berge, K., Burri, L., Berge, R., Stanilov, S., Milanova, A., Penchev, G., Vik, R., Petrov., V., Georieva, T.M., Bivolraski, B. and Georgiev, I.P. (2014) Effect of fish and krill oil supplementation on glucose tolerance in rabbits with experimentally induced obesity.

European Journal of Nutrition, PMID: 25315197

28. Yasui, Y., Hososkawa, M., Mikami, N., Miyashita, K. and Tanaka, T. (2011). Dietary astaxanthin inhibits colitis and colitis-associated colon carcinogenesis in mice via modulation of the inflammatory cytokines. Chemico-Biological Interactions, 193: 79-87.

29. Wibrand, K., Berge, K., Messaoudi, M., Duffaud, A., Panja, D., Bramham, C.R. and Burri, L. (2013). Enhanced cognitive function and antidepressant-like effects after krill oil supplementation in rats. Lipids in Health and Disease, 12: 6.

30. Gigliotti, J.C., Smith, A.L., Jaczynski, J. and Tou, J.C. (2011). Consumption of krill protein concentrate prevents early renal injury and nephrocalcinosis in female Sprague-Dawley rats. Urological Research, 39: 59-67.

31. Ali-Nehari, A., Kim, S-B., Lee, Y-B., Lee, H-Y. and Chun, B-S. (2012). Characterization of oil including astaxanthin extracted from krill (*Euphausia superba*) using supercritical carbon dioxide and organic solvent as comparative method. Korean Journal of Chemical Engineering, 29: 329-336.

32. Tur, J.A., Bibiloni, M.M., Sureda, A. and Pons, A. (2012) Dietary sources of omega 3 fatty acids: public health risks and benefits. British Journal of Nutrition, 107: 23-52

33. Bengtson Nash, S.M., Schlabach, M. and Nichols, P.D. (2014). A nutritional-toxicological assessment of antarctic krill oil versus fish oil dietary supplements. Nutrients, 6: 3382-3402

34. Ulven, S.M., Kirkhus, B., Lamglait, A., Basu, S., Elind, E., Haider, T., Berge, K., Vik, H. and Pedersen, J.I. (2011). Metabolic effects of krill oil are essentially similar to those of fish oil lower dose of EPA and DHA, in healthy volunteers. Lipids, 46: 37-46.

35. Parker, R.W. and Tyedmers, P.H. (2012). Life cycle environmental impacts of three products derived from wild-caught Antarctic krill (*Euphausia superba*). Environmental Science & Technology, 46: 4958-4965.

36. Otton, R., Marin, D.P., Bolin, A.P.A., de Cassia Santos Madcedo, R., Campoio, T.R., Fineto, C. Jr., Guerra, B.A., Leite, J.R., Barros, M.P. and Mattei, R. (2012). Combined fish oil and astaxanthin supplementation modulates rat lymphocyte function. European Journal of Nutrition, 51: 707-718.

37. Faithong, N., Benjakul, S., Phatcharat, S. and Binsan, W. (2010). Chemical composition and antioxidative activity of Thai traditional fermented shrimp and krill products. Food Chemistry, 119: 133-140.

NEWS ITEM

Prime Minister advised the students for high quality research

Prime Minister Modi has urged graduating students to follow the examples set by the doyens of the medical fraternity who were being given lifetime achievement awards at New Delhi in an impressive show held at the AIIMS campus. He expressed hope that society would gain from their work, and the dream of a healthy India would be achieved. The PM further elaborated, India needs to step up medical research, to keep pace with a fast-changing world. Therefore, he urged young doctors to record case-histories in specific areas as they go about their profession. He also urged them to work towards achieving a higher number of publications in scientific journals. He urged graduating students to keep a cheerful attitude towards life, even as they fulfilled their responsibilities as doctors and medical professionals, in all seriousness.

Maithreyi International Visiting Professorship, launched by the Minister of Science & Technology, Dr.Jithendra Singh

The Maithreyi International Visiting Professorship' is an ambitious program, to be jointly supported by the Department of Biotechnology (DBT) and Department of Science and Technology (DST), administered by two aided institutions of the S&T ministry. Dr Jitendra Singh, minister of science and technology recently launched 'Maithreyi International Visiting Professorship', through this unique initiative, each year, 12 leading international scholars in Science and Technology, from across the globe will be invited to visit India. The visiting professors will deliver three talks, one each in a school, a college, and a research institution.

Fight against Tuberculosis. The union ministry of Science and Technology, Department of Biotechnology (DBT), The National Institute of Allergy and infectious diseases (NIAJD, US) and the Office of AIDS Research of the US National Institutes of Health have launched a collaborative project under the indo-US Vaccine action programme to address the growing challenges in controlling tuberculosis in India.

Seminars, Conferences and Workshops

Workshop on 'Biomedical Informatics and Novel Drug Discovery' is being organized by Bioinformatics Centre at Mahatma Gandhi Institute of Medical Sciences, Sevagram during December 1-2, 2014. Interested Medical Teachers, Scientists, Postgraduate and PhD students who intend to apply Interested participants can submit workshop theme-based abstracts (250 words in MS Word format) by email (satishkumar@mgims.ac.in) by November 15, 2014. Last Date of Application: November 15, 2014.

International Conference on Life Science and Engineering (ICLES 2014), Kuala Lumpur, Malaysia. ICLSE 2014, is intended to bring together innovative academics and industrial experts in the field of Life Science and Engineering to a common forum. All papers of ICLSE 2014 will be published in the Journal of Life Sciences and Technologies (JOLST, ISSN: 2301-3672) For important dates and more information, visit www.iclse.org.

International Conference on Environment Science and Biotechnology
(ICESB 2014), Phuket, Thailand 27-28 December, 2014. ICESB-2014 is sponsored by the Asia-Pacific Chemical, Biological & Environmental Engineering Society (APCBEES). For details, see the conference official website.

Bioproceesing India, 2014 meeting, is scheduled from 17th to 20th December, 2014 at ICT, Mumbai. This meeting will bring together eminent scientists from the areas of bioenergy, nutrition as well as healthcare from around the country and provide a platform to discuss the

ongoing research in these fields. This four day meeting would be a great opportunity for undergraduates, graduate students, research scholars, post docs, faculty/scientists and people working in industry not only to present their work but also benefit with the experts from diverse areas in bioprocessing.

Opportunities

IIT, Delhi invites applications from qualified Indian Nationals and Persons of Indian Origins (PIOs) and Overseas Citizens of India (OCIs) for the following positions in the various Departments/ Centres/Schools (in Applied Mechanics, Biochemical Engineering & Biotechnology, Chemistry, Biomedical Engineering, Kusuma School of Biological Sciences, Chemistry, see website for details, http://www.iitd.ac.in/

LG Life Sciences India is offering excellent career opportunities in various disciplines in Life Sciences. Currently offering Business Development Executive posts in Nephrology & Oncology, Infertility. For details, please see the website - http://www.lglsi.com/life_lglsi.html

CSIR, New Delhi - Career opportunities in CSIR National Laboratories as Directors, Scientists at various advanced institutes of CSIR. For more details, visit the website of CSIR – http:// www.csir.res.in/CSIR/External/Heads/ aboutcsir/ announcements/ advt_pp_020514.pdf

IISER, Pune recruiting several project fellows, research fellows in various research projects sanctioned to the institute, for further details, see the website of
http://www.iiserpune.ac.in/links/job-opportunities

NCL, Pune, recruiting various scientific and technical positions. Please see the website for further details - http://www.ncl-india.org/files/ joinus/jobvacancies/ PermanentJobs.aspx? menuid=ql6&childmenustripid=divSubQL6
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Several appointments at inStem – Investigators (Assistant/ Associate/ Full) on renewable contracts and Research Investigators (on a term limited appointment) who are appointments made by the different themes. The application will require your CV, a research statement and contact details of eight referees. The research statement should be framed to align with one of the inStem themes. This may be done following discussions with the theme coordinator and based on the theme project statement on the website. http:// instem.res.in/faculty-positions

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&

8th Annual Convention of Association of Biotechnology and Pharmacy

(Under Golden Jubilee Celebrations of the University)

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* Bioremediation and Environmental Biotechnology
* Nanotechnology and Human Health
* GEMs and GMOs
* Cancer Biology
* Pharmacovigilance of Biopharmaceuticals
* Drug Target Discovery and Development
* Other recent/emerging area theme related to Life Sciences

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