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A Comparative study of iodine value and acid value of Fresh and Used Sunflower oils

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Abstract

The Food value of the edible oils depends on chemical properties like iodine value, rancidity and peroxide value. Thus the determinations of these properties play an important role to ensure the quality of oils. In this study we collect samples of sunflower oil after frying of few objects for 1-2 minutes for three days. With frequent frying of oil Acid value and percentage of rancidity are increased and iodine value is decreased. This may due to the fact that, during frying, the number of free fatty acids are increased and unsaturation is decreased (compound become saturated) leading to rancidity of the oil.

Key Words: Iodine value, Acid value, Rancidity

1. Introduction

Vegetable oil is an important and widely used lipid source for our everyday food products. The food value of the edible oils depends on chemical properties like iodine value, peroxide value, rancidity etc. As oils contain more unsaturation they are very prone to oxidation and become rancid easily. The rancidity of oils depends on the period, temperature and processs of storage (Emily et al., 2008).

Rancidification is the decomposition of fats, oils and other lipids by hydrolysis or oxidation, or both. Hydrolysis will split fatty acid chains away from the glycerol backbone in glycerides. These free fatty acids can then undergo further auto-oxidation. Oxidation primarily occurs with unsaturated fats by a free radical-mediated process. These chemical processes can generate highly reactive molecules in rancid foods and oils, which are responsible for producing unpleasant and noxious odours and flavours. These chemical processes may also destroy nutrients in food. Under some conditions, rancidity, and the destruction of vitamins, occurs very quickly. Polyunsaturated fats such as sunflower, soy and sesame oil are the most unstable and prone to oil rancidity, because of their chemical structures. Oxidative rancidity is the most likely kind of rancidity to affect our food oils. During the process of oxidative rancidity, oxygen molecules interact with the structure of the oil and damage its natural structure in a way that can change its odour, its taste, and its safety for consumption.

The factors which affect rancidity are heat and light. Hence protections from these factors are also important when it comes to our food oils. The chemical composition of oil is also a key factor in the risk of rancidity. Here the basic principles involve saturated and unsaturated fat. The more saturated fat contained in oil, the less susceptible it is to rancidity. The greater the amount of unsaturated fat in an oil, the more likely it is to become rancidity. Since the healthiest plant oils are all highly unsaturated, they are especially susceptible to rancidity. According to a study from the University of Basque Country, the breakdown rate and total formation of toxic compounds depends on the type of oil and temperature. Initially, the oil decomposes into hydroperoxids, and then into aldehydes (Chen et al., 2007). There are many vegetable oils in usage now a days, the commonly used among them is the sunflower oil.

Sunflower oil is the non-volatile oil extracted from sunflower seeds. Sunflower oil is commonly used in food as frying oil, and in cosmetic formulations as an emollient. Sunflower oil is mainly a triglyceride, a typical constituent is shown below(Fig 1). Palmitic acid : 4 - 9%, Stearic acid : 1 - 7%, Oleic acid : 14 - 40%, Linoleic acid : 48 - 74% (Reyes-Hernández et al., 2007)

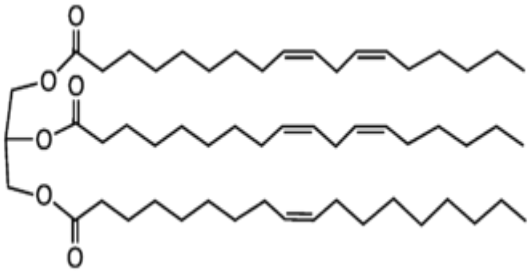

Figure 1.Structure of Sun Flower oil.

Due to the dependance of chemical properties on food value of vegetable oils, determination of iodine value and acid value plays an important role to ensure the quality of oils. K.T Achaya conducted a study on rancidity of Inddian Butterfats (Ghee) (Acharya, 1949). Siddique et al., (2010) conducted a study on physio chemical properties of blends of palmolein with other vegetable oils. Maria et al., (2012) conducted a study on Aldehydes contained in edible oils of a very different nature after prolonged heating at frying temperature.

2. Materials and methods

In this experiment we collect samples of sunflower oil after frying of few objects for 1-2 minutes continuously for three days.The objects used are slices of banana (BN), bitterguard (BG), potato (PT) and pappada (PP). The Iodine value and Acid value of fresh and fried samples were determined and made an attempt to measure extent of rancidity using iodine value.

2.1 Acid value

The acid value is the number of milligrams of potassium hydroxide required to neutralize the free fatty acid in 1 gm. of fat. The acid number, thus, tells us of the quantity of free fatty acid present in a fat.

Reagents used: Oil solvent: Equal volume of 95% alcohol and ether, Potassium hydroxide:0.1N, 2.8g/500ml.

Apparatus used: Burette, Conical flask

2g of oil sample was accurately weighed and suspended in the oil solvent. Added a few drops of Phenolphthalein indicator and titrated against KOH until a faint pink. Color persists for 20-30 seconds. The volume of KOH used was noted. These steps were repeated with blank which does not contain any sample. Calculated the Acid Value by using the following formula,

$$\text{Acid Value} = \text{Titer value} \times N_2 \times 56/\text{Weight of Sample}$$

(Equivalent Weight KOH = 56g, N_2 Normality of KOH=0.0952 N)

2.2 *Percentage of Rancidity*

The extent of unsaturation in oil is normally determined in terms of the iodine value. Thus by comparing the iodine values of the pure sample of the oil and the rancid sample of the same oil we can estimate the extent of rancidity of the oil.

$$\%\ \text{of Rancidity} = (\text{I.V of pure oil} - \text{I.V of rancid oil}) \times 100 / \text{I.V of pure oil}$$

2.3 Iodine value

Iodine number is the number of milligrams of iodine required to completely saturate 100 grams of oil or fat. One application of iodine number is the determination of the the degree of unsaturation of fatty acids. The unsaturation is in the forms of double bonds which react with iodine compounds. The higher the iodine number, the more unsaturated fatty acid bonds are present in an oil. From literatures it is clear that increase in acidity run concurrently with losses in iodine value. Wij's method is used for the determination of iodine value (Jain, 1999). Iodine Value of the different samples were calculated by using the formula,

Iodine Value = [(ml of blank - ml of sample) × Normality of Sodium thiosulphate × 12.69] / Weight of sample in grams. 1 ml of 0.1M sodium thiosulfate solution = 0.01269 g of Iodine.

3. Results and Discussion

3.1. Iodine Value

The Iodine values of fresh and used oils were determined and the results are given in the Table 1.

Days	Iodine value (I_2/100g)			
	Banana (BN)	Bitter gourd (BG)	Potato (PT)	Pappadam (PP)
Day 1	0.6921	0.6662	0.6503	0.6703
Day 2	0.6741	0.6503	0.6345	0.65430
Day 3	0.6421	0.6107	0.6027	0.6222

Table 1. Iodine Value of Sunflower Oil in Different Samples Taken For Three Days

Iodine value of fresh pure sunflower oil = 0.7959

It is observed from the table that iodine value of pure sample is greater than that of fried oil due to the presence of increased number of unsaturated bonds. As a result of consecutive frying, Iodine value decreases due to the decrease in unsaturation, or the oil become saturated. Same trend is shown for all objects.

3.2. Rancidity

From the calculated vlues of iodine value percentage of rancidity is calculated and the results are given in the Table 2.

Days	% of Rancidity			
	Banana (BN)	Bitter gourd (BG)	Potato (PT)	Pappadam (PP)
Day 1	13.041%	16.296%	18.293%	15.780%
Day 2	15.303%	18.293%	20.278%	17.791%
Day 3	19.324%	23.269%	24.274%	21.824%

Table 2. Percentage of rancidity in sunflower oil observed for three days in different samples

It is observed that rancidity increased with frequent frying same results are observed for all objects.

3.3 Acid Value

Acid value of the pure sunflower oil and fried samples were calculated and results are given in Figures 2-5. It is clear from the graphs that acid value increase with number of days for all objects. Acid value of a compound is directly related to number of free fatty acids. As a result of consecutive frying, it is observed that Acid value is increased due to increased number of free fatty acids and which is leading to rancidity of oils.

Acid value of fresh sunflower oil = 0.5031

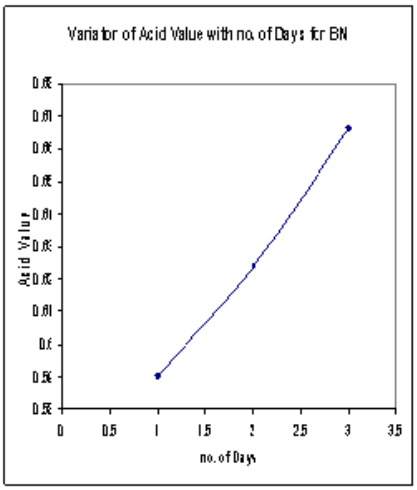

Figure 2

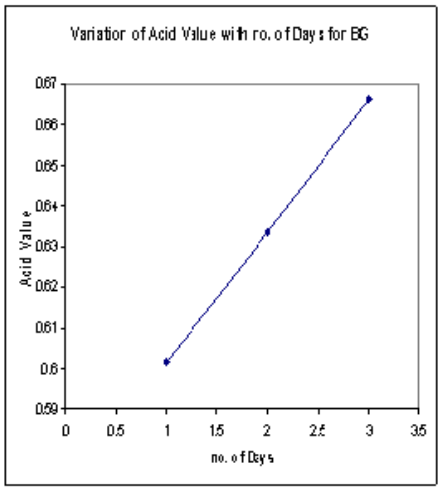

Figure 3

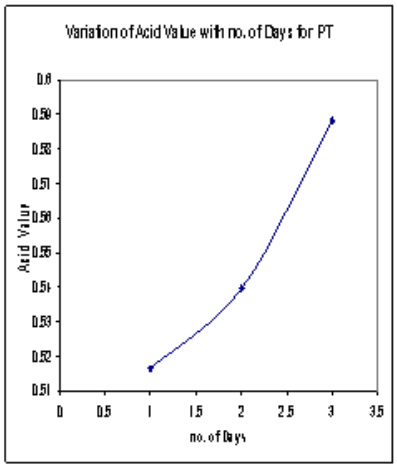

Figure 4

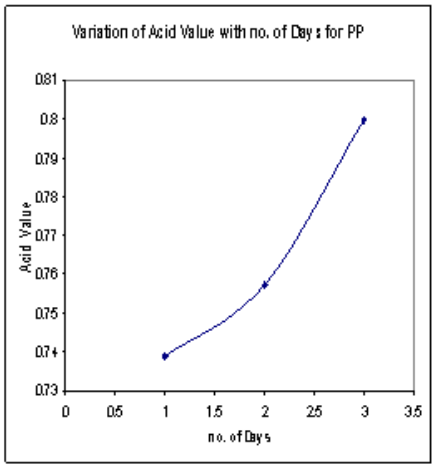

Figure 5

Figure: 2-5 Graphical Representation of Acid values of Fried Samples

4. Conclusions

With frequent frying of oil, Acid value and percentage of rancidity are incerased and iodine value is decreased. Otherwise, increase in acidity run concurrently with losses in Iodine value Acid value of oil is directly related to number of free fattty acids and Iodine value is related to unsaturation. During frying, the number of free fatty acids are increased and unsaturation is decreased (compound become saturated) leading to rancidity of the oil. Thus from the experiment, we can conclude that the chemical properties of oils are affected by heat and time.

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TUMOUR HYPOXIA: A DIRECT GATEWAY TO ANGIOGENESIS AND METASTASIS

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Abstract:

The unique micro-environment within growing tumors characterized by abnormal vasculature results in insufficient supply of oxygen and nutrients to the tumor cells. These hypoxic conditions in tumor induce alterations in expression of genes responsible for further tumor progression and thus hypoxia is associated with a more malignant phenotype and affects genomic stability, apoptosis, angiogenesis and metastasis. HIF-1α, a hypoxia induced transcription factor which regulates the transcription of a battery of genes that are involved in angiogenesis, cell survival, glucose metabolism, invasion, metastasis and apoptosis such as VEGF, MMPs, p53 etc. is up regulated under hypoxia. Eventhough hypoxic cells usually are more aggressive and resistant to conventional cancer therapies such as radiotherapy and chemotherapy; recent studies demonstrated it can specifically be targetted. This review deals with the link between hypoxia and metastasis via angiogenesis.

Key words: Cancer, HIF-1α, hypoxia, metastasis, apoptosis, angiogenesis

1. Introduction:

Death from cancer is most often due to metastasis, a major obstacle in cancer therapy that happens through a series of specific gene mutations and transient changes in gene expression in response to various microenvironments characteristic of tumor cells. Hypoxia is a condition of poor oxygenation and is a characteristic feature of highly proliferating solid tumors. Hypoxia directed metastasis occur through angiogenesis, a process of establishing vascular supply for cancer cells. Tumor vascularization results from low level expression of anti angiogenic factors and over expression of angiogenic factors like VEGF (Hanahan & Folkman, 1996). A key factor regulating the expression of these factors is HIF-1α which is overexpressed in response to hypoxic environment.

The highly proliferating solid tumors rapidly exceed the vascular supply, resulting in a hypoxic acidic microenvironment low in nutrients. To survive under hypoxic environment, tumor cells must adapt to the reduced availability of oxygen and for this they perform many mechanisms like enhanced glucose uptake, glycolysis, survival factor up-regulation (Hockel & Vaupel 2001), vascularization of tissue, etc. As a result of these there occurs a process called angiogenesis which in turn leads to metastasis of tumor cells.

2. Hypoxia inducible factor and its molecular targets:

There is a wide variety of hypoxia-inducible factors (HIFs) which play an essential role in maintaining oxygen homeostasis and primarily mediate adaptive responses to reduced oxygen availability (Kaelin & Ratcliffe 2008). HIFs are heterodimers composed of the subunits, HIF-α and HIF- β. In mammalian cells there are 3 isoforms for HIF-α subunits, HIF-1α, HIF-2α and HIF-3α which are encoded by the genes *HIF-1*, *HIF-2*, and *HIF-3*. Among these, HIF-1 is ubiquitously expressed, whereas HIF-2 and HIF-3 exhibit a more restricted tissue distribution (Semenza, 2001), such that HIF-2α is restricted to endothelial, lung, renal, and hepatic cells (Wiesener et al., 2003), and in tumors of other tissues (Semenza, 2003).

HIF-1 plays a key role in the adaptation of tumor cells to hypoxia by activating the transcription of genes that regulate angiogenesis, cell proliferation and survival, glucose metabolism, pH regulation and migration. Immunohistochemical analysis of human tumor biopsies showed elevated expression of HIF-1α in common cancers (Talks et al., 2000). This protein in its active form controls expression of a battery of target genes such as VEGF (vascular endothelial growth factor), which is crucial for tumor angiogenesis,. glucose transporters 1 and 3 (GLUT1, GLUT3), glycolytic enzymes (aldolase A and C, enolase 1, hexokinase 1 and 3, lactate dehydrogenase A, phosphofructokinase L and phosphoglycerate kinase 1) and IGF2 (insulin-like growth factor 2), promoting tumor cell survival (Semenza 2002). Because of its crucial role in tumor survival, loss of HIF-1 negatively affect tumor growth.

HIF-1β is constitutively expressed, while the expression of HIF-1α is controlled to low levels in normoxic condition. This negative regulation is mediated by oxygen dependent HIF- 1α proteasomal degradation where oxygen dependent prolyl hydroxylase modifies proline residues at 564 and 402 positions and helps binding of von-Hippel-Lindau tumour suppressor protein (Epstein et al., 2001). This protein can act as recognition factor for E3 ubiquitin protein ligase targeting HIF-1 α for proteasomal degradation (Jaakkola et al., 2001). Under hypoxic condition,

the low oxygen level limits the ubiquitination of HIF-1α via limiting the action of prolyl hydroxylase (Epstein et al., 2001) and thus hypoxia induces over expression of HIF-1α.

Under hypoxic conditions HIF-1α become stable and is directed to the nucleus by specific basic sequences at the C-terminal end which act as nuclear localization signal (Kallio et al., 1998). In nucleus they heterodimerizes with constitutively expressed HIF-1β and the resultant HIF complex becomes transcriptionally active (Kallio et al., 1997). Once activated HIF complex interacts with 50 base pair cis-acting HRE (hypoxia response element) located in the regulatory regions of target genes and also with transcriptional coactivators like general transcription activator p300 (CBP), the molecular chaperone HSP90 and, Von Hippel Lindau (VHL) tumour suppressor (Maxwell et al., 1999).

2.1 Hypoxia directed angiogenesis

There is a large number of hypoxia regulated genes which are essential for angiogenesis and leading to metastasis such as VEGF, IL-8, angiogenin, FGF and PDGF angiopoeitin (Ang-2) (Giordano & Johnson 2001; Koong et al., 2000). The expression of Vascular endothelial cell growth factor (VEGF) is regulated in an oxygen dependent manner and it induces endothelial cell proliferation and directly participates in angiogenesis by recruiting endothelial cells into hypoxic and avascular area and stimulates their proliferation (Conway et al., 2001). Expression of VEGF and its receptors like flt-1 (VEGFR-1) and flk-1/KDR (VEGFR-2) are reduced in normoxic condition whereas overexpression occurs in hypoxia (Plate et al., 1992)

HIF-1α is the major transcription factor in hypoxia induced VEGF gene expression (Forsythe et al 1996). Hypoxia induced overexpression of VEGF also rely on its mRNA stability (Shima et al 1995). 3' UTR of VEGF gene has 5 AU rich hypoxia-inducible RNA-protein binding sites and when HuR, a RNA binding protein, specifically binds to this region the VEGF mRNA stability increases (Levy et al., 1998).

HIF-1 activity upregulate angiogenic factors and decrease expression of angiogenic inhibitors such as thrombospondin (Dewhirst et al., 2008).

2.2 *Hypoxia and metastasis*

Metastasis is a complex multistep process in which tumor cells detach from primary tumor travel through blood stream or lymphatic system to lodge themselves at a distant site to form secondary tumor. Many hypoxia related angiogenic factors and cytokines has potential role in metastasis. These include interleukin-8 (IL-8), platelet-derived endothelial cell growth factor (PD-ECGF),

and basic fibroblast growth factor (bFGF) (Slavin 1995). Over expression of angiogenic factors in tumor lead to increased survival of tumor growth at distant metastatic site allowing their proliferation to form macroscpic metastatic nodules (Fidler & Ellis, 1994). Hypoxia induced angiogenesis and metastasis results in development of vascular spots which promote tumor cell intravasation, in turn increasing the probability of metastasis (Rofstad & Halsor, 2002).

Metastasis includes invasion and migration through various cell barriers and extracellular matrix (ECM) by proteolytically degrading barrier components. This degradation is principally carried out by family of proteins like MMPs (matrix metalloproteinase), adamalysin-related membrane proteinases, tissue serine proteinases, including tissue plasminogen activator, urokinase, and plasmin. These proteases cause release of growth factors from the ECM to assist growth of tumour cells at the new site (Andreasen et al., 2000). Among these proteins MMPs that can degrade all the constituents of basement membrane and structural components of the stroma is upregulated in response to hypoxia (Subarsky & Hill, 2003).

Other hypoxia-regulated genes which are influential in tumor invasion and metastasis include extracellular matrix proteins like LOX (Lysyl oxidase), CTGF (Connective tissue growth factor), E-cadherine, CXCR4/SDF-1, MIF (migration inhibitory factor), etc. LOX, important in cell invasion and migration through regulation of focal adhesion kinase activity, regulate key steps in the metastatic process, including extracellular matrix interactions, invasion, migration, and distant metastatic growth (Saikumar et al., 1998). CTGF function in tumor metastasis by supporting anchorage-independent cell growth. E-cadherine is a transmembrane protein which play critical role in epithelial cell-cell adhesion and its expression is negatively regulated by HIF-1α (Li et al., 1997).

2.3 Hypoxia and apoptosis

Apoptosis, programmed cell death, is under control of various tumor suppressor genes and oncogenes. Hypoxia usually favors apoptosis by many mechanisms. They include MPT (mitochondrial membrane transition) induction which results in release of Cytochrome c to the cytoplasm, which in turn interact with Apaf-1 (apoptotic protease activating factor-1), a central kinase in apoptosis signalling. This molecule then activate caspase 9 which in turn activate caspase 3, 6 etc leading to cell death (Li et al., 1997). Another mechanism is through the action of Bax gene, a member of Bcl-2 family. Bax support cytochrome c induced apoptosis by making pores in the outer mitochondrial membrane. Hypoxia limits energy production in mitochondria. This reduction in ATP production leads to activation of BAX gene (Saikumar et al., 1998).

Hypoxia induced apoptosis may also occur through generation of free radicals (ROS). Studies have shown that hypoxia stabilizes p53 protein thus promoting apoptosis and it mainly occurs through the mediation of HIF-1α. Phosphorylation of HIF-1α is critical here, such that dephosphorylated form exerts pro-apoptotic effects under hypoxia where as its phosphorylated form does not (Suzuki et al., 2001).

Hypoxia can also lead to cell survival. It was shown that hypoxia can activate Akt signaling pathway which play major role in signal transduction responsible for cell survival and proliferation. Akt (a serine/threonine kinase) decreases activity of pro-apoptotic factors (Bad, Bid, Bax, Bak) by phosphorylation and exhibit opposite effect on anti apoptotic factors (Bcl-x1, Bcl-2) (Mayo & Donner, 2002). This pathway also induces translocation of Mdm-2 into nucleus which targets p53 for proteasomal degradation (Kunz & Ibrahim, 2003). Akt pathway also inhibits action of caspase 9 (Kunz & Ibrahim 2003), and thus make cell resistant to apoptosis under hypoxia. Hypoxia also prevents Bax from accumulating in the mitochondria thus maintaining integrity of mitochondrial membrane in turn making cell resistant to apoptosis.

Taken together, hypoxia select for cells which are more resistant to apoptosis and more aggressive and decrease the responsiveness to treatment.

3. Hypoxia Targeted Cancer therapy

Hypoxia makes major complications in conventional cancer therapies such as radiation therapy and chemotherapy. The presence or absence of molecular oxygen is known to influence the biological effect of ionizing radiation; cells obtain radioresistance under hypoxic conditions (Brown & Wilson, 2004). Radiation chemical studies have elucidated that the depletion of oxygen results in the inefficient formation of DNA strand breaks by ionizing radiation and prevents the damage from being repaired and HIF-1 plays a pivotal role in hypoxia-related tumor radioresistance (Moeller & Dewhirst, 2006). Hypoxia also contributes for resistance to chemotherapy. This happens mainly because most fractions of drugs do not reach tumor mass as they are located far away from blood vessels. Also, as they have reduced metabolic rate, are unaffected by drugs which acts upon actively proliferating cells. Unfortunately most of the drugs which are in current use acts on actively proliferating cells.

However hypoxia differentiates tumor cells from normal cells and so can be exploited for cancer therapy. Gene therapy is one among them which target hypoxia for tumor treatment. It affects both primary tumor and metastases (Dachs & Tozer, 2000). Here the transgene expression will be restricted to hypoxic cells. It has been demonstrated that expression of an enzyme normally

absent in human body under hypoxic- response promoter could convert a nontoxic prodrug into toxic form in tumor condition. Proof can be obtained from enzyme- prodrug therapy done previously using *E coli* cytosine deaminase (Dachs, 1997). It catalyzes the conversion of nontoxic 5- flurocytosine to toxic 5- fluro uracil. To attain significant antitumor activity gene delivery should be done to maximum number of tumor cells which can be attained by genetically engineered macrophages which are not killed by most active drugs and can accumulate in hypoxic regions (Griffiths et al., 2000).

Other hypoxia targeted cancer therapy includes use of hypoxic cytotoxins like TPZ (Tirapzamine) (Brown, 2002). Once inside the cell they get reduced by reductase to form highly reactive radical. Under normoxic conditions these are oxidised back to relatively nontoxic parent and super oxide radical limiting normal cell damage. There are other drugs such as mitomycin C, AQL4N, etc. (Patterson, 1993) which are toxic to hypoxic cells.

4. Conclusion:

Hypoxia induces expression of several genes which are responsible for tumor progression. HIF-1α is a hypoxia induced transcription factor which is stabilized in the absence of oxygen. Other genes which are induced by hypoxia include genes responsible for angiogenesis, metastasis, and apoptosis. Hypoxia regulated apoptosis is controversial, but it has been demonstrated that hypoxia enrich for highly metastasizing tumor cells. Thus hypoxia becomes major hindrance in conventional cancer therapy. Advanced cancer therapy strategies target hypoxia to develop treatment methods which are specific to tumor cells.

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Isolation, screening and selection of Poly-β-Hydroxybutyric Acid producing bacteria from soil

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Abstract

Synthetic plastics are of petrochemical origin and are non-degradable. They cause waste disposal problems and leads to environmental pollution. Bioplastics (polyhydroxyalkanoates or PHA) are of biological origin and could be used as substitutes for petroleum derived synthetic plastics because of their similar physical and chemical properties. They are completely biodegradable too. In the present study, an attempt was made to isolate efficient PHB producing bacteria from soil. PHB accumulators were screened based on viable colony staining method using Sudan Black B and were subjected to quantitative estimation of PHB. From the selected promising bacterial isolates, one deep PHB accumulator was selected and identified. The morphological, biochemical characteristics and colony characteristics of the organism suggested that it may be a Bacillus species. Further molecular studies are needed for taxonomic levels of identification. The studies on the effect of different carbon sources on the PHB production by the selected bacteria, by analyzing the concentration of PHB produced indicated that maltose and sucrose favored maximum accumulation of PHB.

Key words: Polyhydroxyalkanoates, PHB, Plastics, Sudan Black

1. Introduction

Plastics play a major role in our everyday lives. However, these oil-based polymers take many years to degrade, which poses a serious environmental problem. In response to problems associated with plastic waste and its effect on the environment, there has been considerable interest in the development and production of biodegradable plastics. Among the various biodegradable polymer materials, polyhydroxyalkanoates (PHA's) are prospective substitutes for conventional petrochemical plastics because of their similar properties to various thermoplastics and elastomers, and complete degradability upon disposal under various environments. PHAs are polyesters that accumulate as inclusions in a wide variety of bacteria such as *Azotobacter, Bacillus, Archaebacteria, Methylobacteria, Pseudomonas, Haloferax, Vibrio, etc*.

PHB, a member of the PHA series, is a biodegradable and biocompatible thermoplastic produced by various microorganisms as a carbon or energy storage material and is readily biodegradable. It could be used in food packaging, plastic films, surgical sutures, controlled drug delivery, etc (Fatemeh & Ebrahim, 2003).

The present study involves the screening of bacterial isolates for PHB accumulation and preliminary studies for optimizing the cultural condition for PHB accumulation in a selected strain of bacteria.

2. Materials and methods

2.1. Chemicals

All the chemicals used in this study were of analytical grade procured from reputed Indian manufacturers.

2.2. Collection of samples

Soil samples of different consistency were collected from various sources and used for the isolation of bacteria. Dry sand, damp garden soil, dry garden soil, Plant organic matter rich soil and animal organic matter rich soil were taken for the isolation of PHB producing bacteria.

2.3 Isolation of bacteria from different samples

1g of soil sample collected were serially diluted in sterile distilled water and plated on nutrient agar medium supplemented with 1% glucose and incubated at room temperature for 24-48 hrs. The predominant bacterial colonies were picked up, and preserved on nutrient agar slants till further use.

2.4. Preparation of crotonic acid standard curve

Standard curve of PHB was prepared following the method of Slepecky & Law (1961). Pure PHB when dissolved in 10 ml concentrated H_2SO_4 and heated for 10 minutes it will get converted into crotonic acid. Various concentrations of crotonic acid were prepared in concentrated H_2SO_4 and absorbance was read at 235 nm against a sulfuric acid blank. Standard graph was plotted between concentration of crotonic acid and OD at 235 nm.

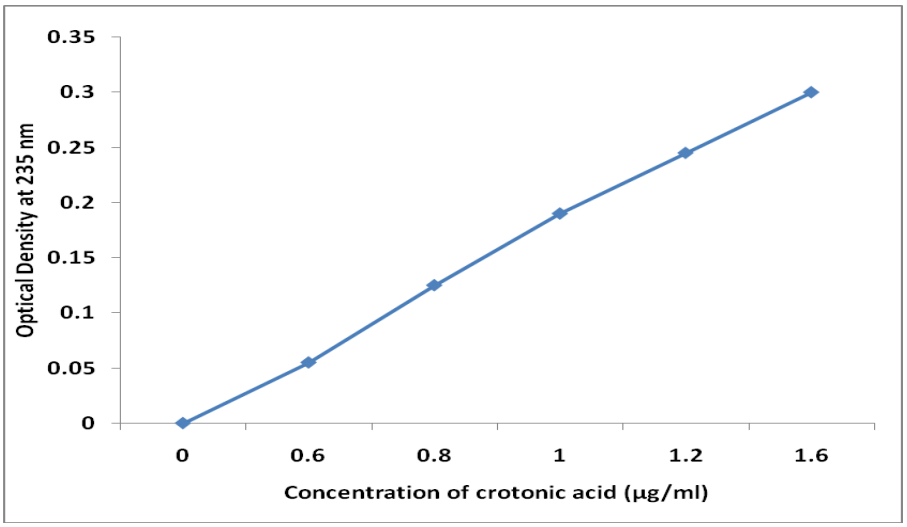

Figure 1. Crotonic acid standard curve

2.5. Rapid screening of native bacterial isolates for PHB production

For rapid screening of PHB, all the bacterial isolates were tested for PHB production by viable colony method of screening using Sudan black B stain (Juan et al., 1998). Nutrient agar medium supplemented with 1% glucose was sterilized and cooled to 45^{o}c. The medium was poured in to sterile petriplates and allow to solidification. The plates were taken and divided in to 4 equal parts and in each part a bacterial isolated was spotted. The plates were incubated at room temperature for 24-48 hrs. After incubation, ethanolic solution of Sudan Black B solution (0.02%) was spread over the colonies and the plates kept undisturbed for 30 minutes. They are washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue colonies were taken as positive for PHB production. All the positive isolates were assigned by code numbers based on their source of isolation. The assays were done in duplicates.

2.6. Quantification of PHB production in selected isolates

2.6.1. Quantification of PHB production in selected isolates: by isolating and quantifying PHB

All the Sudan Black B positive isolates were subjected to quantification of PHB production as per the method of John & Ralph (1961). The representative bacterial colonies inoculated in to 5 ml of nutrient broth and incubated for 24 hr at room temperature. After incubation the bacterial cells containing the polymer were pelleted at 1500 rpm for 10 min, and the pellet washed with saline and recentrifuged to remove the unwanted materials. The pellet was suspended in equal volume of sodium hypochlorite (2 ml) and chloroform (2 ml) and the mixture incubated at room temperature for 24 hr. The whole mixture was again centrifuged and 0.5 ml of chloroform layer separated by using micro pipette without disturbing the mixture. The polymer granules were dissolved in hot chloroform. The test tubes were incubated to evaporate the chloroform after that concentrated 10 ml H_2SO_4 was added, and boiled at100^o C for10 minutes. The addition of sulfuric acid converts the polymer into crotonic acid which is brown coloured. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. By referring to the standard curve, the quantity of PHB produced was determined. The assays were done in duplicates.

2.6.2. Quantification of PHB production in selected isolates: by Sudan black staining of broth culture

The 13 organisms isolated were inoculated in to the test tubes which containing 3 ml of nutrient broth media and incubated at room temperature for 24 hours. Each assay was done in duplicates. After incubation, Sudan black solution (0.02%) added to the tube and kept undisturbed for 30 minutes. After that the tubes were centrifuged at 1500 rpm for 10 minutes, the stained cells were washed with sterile saline and the pellet suspended in saline was read at 600 nm using cell pellet suspended in saline without the Sudan black staining as blank. During incubation, the cells having PHB granules will take up the Sudan black and the colour could be measured using a calorimeter at 600 nm.

2.7. Characterization of the selected PHB producing bacterial isolate

The selected, most efficient PHB producing bacterial isolates were subjected to a set of morphological, physiological and biochemical tests for the purpose of identification.

2.7.1. Morphological tests

The thirteen potent PHB accumulating strains were examined for their colony morphology, cell shape and gram reaction as per the standard procedures given by Anon (1957) and Barthalomew & Mittewer (1950).

2.7.1.1. Colony characterization

The colony characters viz., shape, colour, elevation, surface appearance, etc were observed on Nutrient agar medium.

2.7.1.2. Gram staining

Twenty four hour old culture was smeared on a clean glass slide and heat fixed. The smear was covered with crystal violet for 30 seconds and washed off with 95 per cent ethyl alcohol. The slide was washed with distilled water and drained. Safranin was applied on smear for 30 seconds as counter stain, washed with distilled water and dried. The slide was observed under microscope for gram reaction.

2.7.1.3. Fluorescent staining method

Detection of PHB production in the isolates was also done following fluorescent staining method using acridine orange. 2 ml of culture of the isolates was transferred in to a eppendorf tube containing acridine orange and incubated for 30 minutes at 30^0C. After incubation period, the culture was centrifuged at 4000 rpm for 5 minutes. The pallet was collected and resuspended in distilled water. A smear was prepared on a clean glass slide and observed under microscope at 460nm. The appearance of yellow coloured granules inside the cell indicated PHB production.

2.7.1.4. Endospore Staining

One drop of sterile saline water was taken on a clean glass slide for spore staining. A loopful bacterial old slant culture was taken in the drop and smear was made on the slide. The film was dried over flame gentle heating. The slide was then placed over a beaker and 5% malachite green was added drop wise on the slide. Boiling of the malachite green was avoided by adding more malachite green. The slide was taken out of the stream and washed gently with tap water. The preparation was stained with safranin solution for 1 min and washed with gentle stream of tap water, and placed under immersion lens with immersion oil.

2.7.1.5. Motility test

This method is used to detect the ability of microorganism to move by itself. Organisms are observed in a drop that is suspended under a cover glass in a concave depression slide. The slide for a hanging drop is ground with a concave well in the center; the cover glass holds a drop of the suspension. When the cover glass is inverted over the well of the slide, the drop hangs from the glass in the hollow concavity of the slide. Since the drop lies within an enclosed glass chamber, drying out occurs very slowly. A ring of Vaseline around the edge of the cover slip keeps the slide from drying out. By observing under microscope the motility can be seen.

2.7.2. Biochemical tests

Biochemical tests were carried out as per the method given by Cappuccino & Sherman (1992) with 24 hr old cultures.

2.7.2.1. Catalase test

Nutrient agar slants were inoculated with overnight grown test organisms and were incubated at 30^oC for 24 hr. After incubation, one loop full of organism placed on the glass slide and flooded with one ml of hydrogen peroxide and observed for gas bubbles. The occurrence of gas bubbles was taken as positive for catalase test.

2.7.2.2. Oxidase test

A strip of filter paper, soaked in the oxidase reagent is placed in a Petri dish and the colony to be tested is placed on the paper. In a reaction the area of paper turns dark.

2.7.2.3. Indole production test

To the pre sterilized peptone water, the test cultures were inoculated. The tubes were incubated for24- 48 h at 37^oC.this test demonstrate the production of indole from tryptophan. After incubation, each tube was added with ten drops of Kovac's reagent. The production of red colour was taken as positive for the indole production.

2.7.2.4. Methyl red test

This test is employed to detect the production of acid during the fermentation of glucose. Five drops of solution of methyl red are added to the culture in glucose phosphate medium and incubated at 37^{o}C for 24 hr. Red colour is taken as positive while yellow colour signifies negative reaction.

2.7.2.5. Voges Proakauer test (VP)

This test depends on the production of acetyl methyl carbinol from pyruvic acid. This test is performed by adding α naphthol and 40% KOH to the phosphate medium culture of the organism incubated at 37^{o}C for 24-48 hr. In a positive test pink colour appears within 2-5 minutes, deepening in to magenta or crimson in half an hour. In a negative reaction, it remains colour less for half an hour.

2.7.2.6. Citrate utilization test

Citrate utilization test was performed to find out the ability of the bacterial isolates to utilize or ferment citrate as the sole source of carbon. It was done on the Simmon's Citrate Agars slants and a change in the colour of the medium from green to blue was positive for the test.

2.7.2.7. Nitrate reduction test

This test is done in the broth culture which containing1%KNO3 it incubated at 37^{o}C for 24 hr and add equal volumes of solutions sulphanilic acid and α naphthalamine .The test reagent added to the culture. A red colour developing within few minutes signifies positive reaction, while absence of colour indicates a negative reaction.

2.8. Effect of different carbon source on PHB production by the Selected Bacterial isolate

The selected bacterial isolates were grown in test tubes containing 5 ml nutrient broth with different carbon sources viz., glucose, fructose, sucrose, maltose, starch, glycerol, coconut oil, sodium acetate and trisodium citrate at 1 per cent level. The tubes were incubated at room temperature for 24 hours. After incubation, PHB produced by the isolates were quantified spectrophotometerically by the method of John & Ralph (1961).

Briefly, after incubation the bacterial cells containing the polymer were pelleted at 1500 rpm for 10 min, and the supernatant discarded. The pellet was suspended in equal volume of sodium hypochlorite and chloroform (2 ml) and the mixture incubated at room temperature for 24 hr. The whole mixture was again centrifuged and 0.5 ml of chloroform layer separated by using micro pipette without disturbing the mixture. The polymer granules were dissolved in hot chloroform. The test tubes were incubated 24hr to evaporate the chloroform after that concentrated 10 ml H_2SO4 was added, and boiled at 100^{o} C for10 minutes. The addition of sulfuric acid converts the polymer into crotonic acid which is brown colored. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. By referring to the standard curve, the quantity of PHB produced was determined. The assays were done in duplicates.

3. Results and Discussion

3.1. Isolation of Bacteria from various soil samples

Five different soil samples (dry sand, dry garden soil, damp garden soil, animal organic matter rich soil and plant organic matter rich soil) were collected and about 41 bacteria were isolated on nutrient agar fortified with glucose (1%), purified and maintained as pure cultures. All the isolates were given code numbers based on the soil of origin.

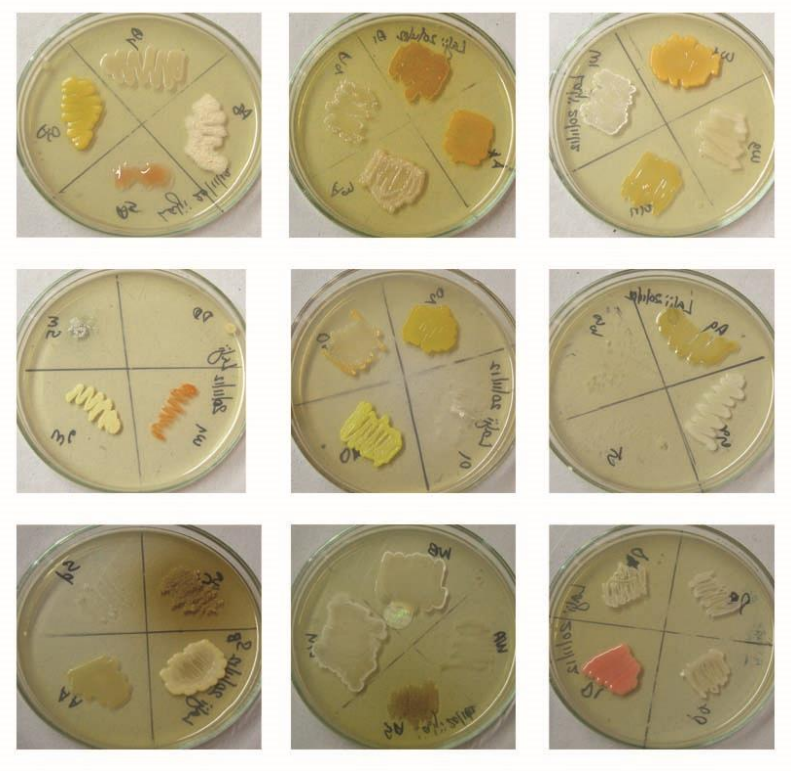

Figure 2. Colonies of isolates from various soil samples

3.2. Screening of the Isolates for PHB Production

All the 41 isolates were subjected for visual screening for PHB production using Sudan black B. It was observed that out of 41 isolates, 13 accumulate PHB (Figure 3).

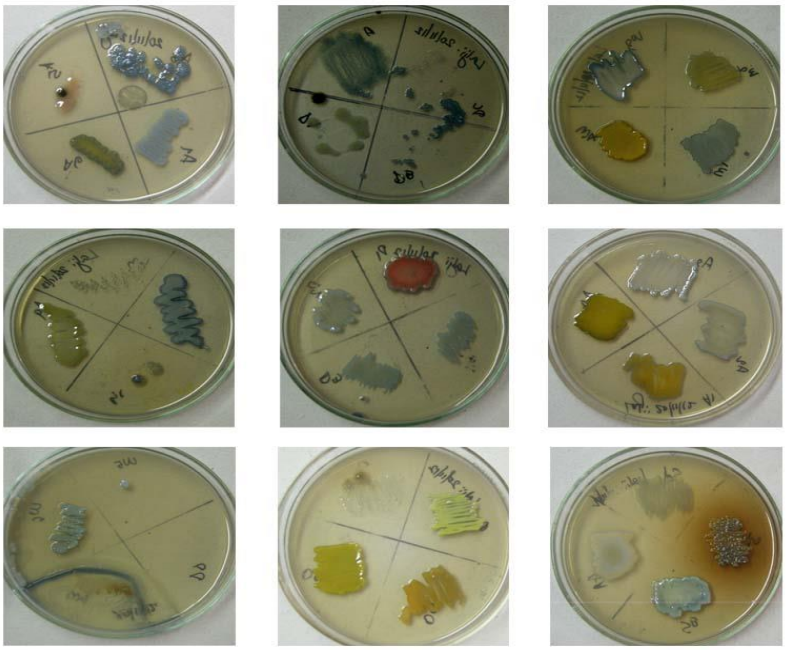

Figure 3. Screening of the Isolates for PHB Production by Sudan black staining

- 4 isolates out of 7 isolated from dry sand accumulated PHB.
- 3 isolates out of 8 from dry garden soil accumulated PHB.
- 3 isolates out of 10 from damp garden soil accumulated PHB

- No isolates out of 4 from plant organic matter rich soil accumulated PHB
- 3 isolates out of 12 from animal organic matter rich soil accumulated PHB
-

3.3. Selection of high level PHB accumulator from the screened isolates

3.3.1. Selection of high level PHB accumulator from the screened isolates – Sudan Black staining of broth culture

When the 13 isolates were screened by Sudan Black staining of broth culture, a few isolates (9 Nos) showed maximum absorbance compared to others indicating maximum staining of cells which was further indicative of higher level accumulation of PHB in these isolates. The isolates that showed maximum accumulation are given in table 1.

The data given in table 1 indicated that the isolates S1, S2, S5, S6, W1, W3, W9, A8 and D6 gave maximum PHB accumulation as shown by Sudan black staining of cells.

Isolate	OD at 600 nm
S_1	**0.99**
S_2	**0.62**
S_5	**0.86**
S_6	**0.80**
W_1	**0.71**
W_3	**0.68**
W_9	**1.23**
D_6	**0.79**
A_8	**0.59**

Table 1. Selection of high level PHB accumulator from the screened isolates by *Sudan Black staining of broth culture*

3.3.2. Selection of high level PHB accumulator from the screened isolates – Quantitation of Isolated PHB using crotonic acid standard graph

The level of PHB was quantitated by isolating from broth cultures and using crotonic acid standard curve. The results showed that the isolate from S_2 contained about 0.221 µg of PHB equivalent crotonic acid /ml. It may be due to the dry granule like growth and appearance of S2 in broth that lead to a comparatively lesser calorimetric reading after sudan black staining.

3.3.3. Selection of high level PHB accumulator from the screened isolates – Sudan Black staining of Agar culture

In figure 4, the rightmost one is isolate S2, which was selected for further studies since it was the deepest accumulator of PHB, maintained as pure culture and used for further studies.

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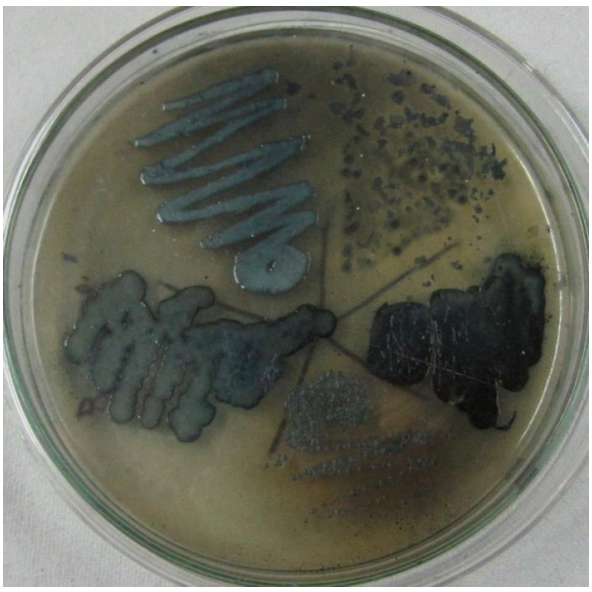

Figure 4: Five deep accumulators as seen after Sudan Black staining.

3.4. Characterizations of the selected PHB accumulator isolate (S2)

3.4.1. Morphological tests

- **Colony characteristics**

White**,** round opaque, irregular colonies were seen on nutrient agar medium

- **Gram Staining**

Purple coloured thick rod shaped bacilli were seen under microscope, indicating Gram positive bacteria.

- **Fluorescent staining method**

The appearance of yellow coloured granules inside the cell was seen after acridine orange staining when observed under a flourescent microscope and this indicated PHB accumulation inside the cell.

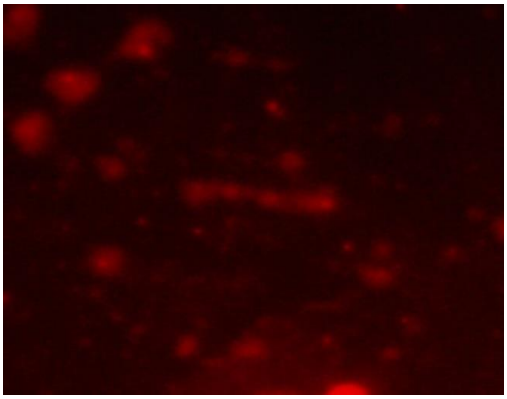

Figure 5. Bacteria showing PHB granules following acridine orange staining. Yellow coloured granules inside the cell indicate PHB.

- **Endospore staining**

On staining with malachite green, the organism was found to contain spores which were seen in green colour and the organism was red in colour.

- **Hanging Drop Test**

Motile rods were seen under microscope indicating that the isolate is a motile organism.

3.4.2. Biochemical Tests

This organism was indole negative, methyl red negative, voges proskauer negative, citrate negative, nitrate negative, TSI positive, catalase positive, oxidase negative and fermented most of the carbohydrates glucose, maltose, mannose, lactose and fructose with the production of acid but no gas production. The results are given in Table 2.

Tests	**Results**
Colony morphology	White, round, raised, irregular colony
Cell shape	Rod shape
Gram's staining	Gram positive bacilli
Spore staining	Positive
Motility Test	Positive
Catalse Test	Positive
Oxidase Test	Negative
Indole Test	Negative
Methyl Red Test	Negative
Voges Proskauer Test	Negative
Citrate utilization Test	Positive
Triple sugar iron agar Test	Positive, acid slant, alkaline butt, no gas
Nitrate reduction Test	Negative

Tests	**Results**	
Carbohydrate utilization	Acid production	Gas
Glucose	+	-
Maltose	+	-
Mannose	+	-
Lactose	+	-
Fructose	+	-

Table 2. Biochemical Test results of the selected isolate (S2) that gave maximum yield of PHB

The biochemical characteristics and colony characteristics of the organism suggested that it may be a Bacillus species. Further molecular studies are needed for further taxonomic levels of identification.

3.5. Effect of different carbon sources on PHB production by isolated organism

Carbon source	Concentration equivalent to crotonic acid (µg/ml)
Starch	1.6
Glycerol	1.48
Glucose	1.36
Coconut oil	0.70
Fructose	0.26
Sucrose	**1.75**
Trisodium citrate	1.53
Sodium acetate	1.6
Maltose	**1.75**

Table 3. Effect of different carbon sources on PHB production by the selected bacteria

The studies on the effect of different carbon sources on the PHB production by the selected bacteria, by analyzing the concentration of PHB produced indicated that maltose and sucrose favored maximum accumulation of PHB (Table 3).

Plastics have become an important part of modern life and are used in different sectors of daily life operations for packaging, as building materials, in consumer products and many more. Most of the plastics and synthetic polymers are produced from petrochemicals. Because of their persistence in the environment and nonbiotic origin, use and accumulation of plastics has caused several serious and deleterious effects on environment – the biotic and abiotic aspects of environment are being affected. Plastic bags or sheets do not allow water and air to percolate into earth causing reduction in fertility status of soil, depletion of underground water sources and damage to animal life, and so on.

PHAs such as PHB have been considered to be good candidates for biodegradable plastics and are mainly produced by microbial fermentation processes. Many studies are being focused on the efficient production of PHB based polymers with desirable material properties by bacteria from different habitats or by recombinants. Earlier studies on PHB production by several bacteria suggested the need for developing a new culture medium and cultivation strategy in order to achieve increased volumetric productivity.

5. Conclusions

In the present study, an attempt was made to isolate efficient PHB producing bacteria from samples collected from various soils. Dry sand was found to contain more PHB producers compared to other soil samples. The biochemical characteristics and colony characteristics of the organism suggested that it may be a Bacillus species. Further studies are needed to be

done for confirming this result. Studies indicated that maltose and sucrose favored maximum accumulation of PHB by this selected bacteria compared to other tested carbon sources.

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Study on Biosurfactant Properties of Bacterial Isolates from Oil Contaminated Sites

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Abstract

One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Accidental release of petroleum products are of particular concern with the environment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants . Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost effective and will lead to complete mineralization. Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds and cell protein or transformation of complex organic contaminants to other simple organic compounds by biological agents like microorganisms. Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants and most species have the ability to produce a large variety of surface-active materials or surfactants. Hydrocarbon contaminated sites are the most promising for the isolation of biosurfactant producing microbes. Biosurfactants are often produced by bacteria capable of growing on hydrocarbons and have been shown to stimulate the growth of these bacteria and to accelerate bioremediation. The aim of the study was to screen the efficient bacteria capable of utilizing vegetable oil, diesel and engine oil as their soul carbon and energy source and their biosurfactant property. The biodegradation of oil contaminated soils, which exploit the ability of microorganisms to degrade or detoxify organic contamination; has been established as one of the efficient, economic versatile and environmentally sound treatment.

Key words: Indigenous, Biosurfactant , Contamination

1. Introduction

Biodegradation is a large constituent of oil weathering and is a natural process whereby bacteria or other microorganisms alter and break down organic molecules into other substances, eventually producing fatty acids and carbon dioxide (Hoff, 1993). The initial steps in the biodegradation of hydrocarbons by bacteria and fungi involve the oxidation of the substrate by *oxygenases*, for which molecular oxygen is required. Alkanes are subsequently converted to carboxylic acids that are further biodegraded via ß-oxidation (the central metabolic pathway for the utilization of fatty acids from lipids, which results in formation of acetate which enters the tricarboxylic acid cycle). Aromatic hydrocarbon rings generally are hydroxylated to form diols; the rings are then cleaved with the formation of catechols which are subsequently degraded to intermediates of the tricarboxylic acid cycle. Interestingly, fungi and bacteria form intermediates with differing stereochemistries. Fungi, like mammalian enzyme systems, form trans-diols, whereas bacteria almost always form cis-diols.Since bacteria are the dominant hydrocarbon degraders in the marine environment, the biodegradation of aromatic hydrocarbons results in detoxification and does not produce potential carcinogens. The complete biodegradation (mineralization) of hydrocarbons produces the non-toxic end products carbon dioxide and water, as well as cell biomass (largely protein) which can be safely assimilated into the food web (Atlas, 1995) Petroleum bioremediation is carried out by microorganisms capable of utilizing hydrocarbons as a source of energy and carbon (Reisfeld A et all., 1972;Ron EZ et all.,2000;Rosenberg E et all.,1998).

These microorganisms are ubiquitous in nature and are capable of degrading the various types of hydrocarbons Therefore, it is not surprising that bacteria growing on petroleum usually produce potent emulsifiers. These surfactants help to disperse the oil, increase the surface area for growth, and help detach the bacteria from the oil droplets after the utilizable hydrocarbon has been depleted (Rosenberg E, 1993).

The oils derived from plants usually extracted from seeds are important products used pharmaceutically, industrially and nutritionally.(P.PrajaktaKamble and A.Avinash Raut,2011)Many vegetable oils are used directly as ingredients in food as it has various properties like; high caloric value, it makes the ingredient stick less to each other ;it can also carry flavours to other ingredients in food (potter N.N and Hotchkiss J.H , 1995).In pharmaceutical industries vegetable oils are used for preparing concentrated solutions of vitamin A and D. The ground nut oil emulsion has been used successfully for the control of many insect pets of plants. Many vegetable oils are used to make soaps,candles,perfumes,etc. Thus the production, use and transportation of vegetable oil is growing fast around the world. The chocking up of sewage lines because of oily wastes of vegetable origin generated from house, canteens, army bases,pharmaceutical industries has been increased resulting in foul smell and backup problems. Hence there is a need for micro organisms capable of degrading vegetable oil wastes (santhiniK.mylaj,2009)

Engine oil is a complex mixture of hydrocarbons and other organic compound, including some organometallic constituents(Butter and Mason,1997) It is mainly comprising 1) Alkyl benzene having carbon atom.2) Antioxidant 3)Extreme pressure additive in the range 0.01 - 0.05% by weight 4)Antifoaming agent in the range of 0.01 to 1.0% by weight. Engine oil is used to lubricate the parts of an automobiles engine. The most important characteristic of the engine oil for automotive use is its viscosity. New motor oil contains a higher percentage of fresh and lighter hydrocarbons that would be more of a concern for acute toxicity to organisms . Used motor oil contains more metals and heavy polycyclic aromatic hydrocarbons that would contribute to chronic hazards including mutagenicity and carcinogenicity (Keith and Telliard,1979)

Diesel fuels are complex mixture of saturated hydrocarbons and aromatic hydrocarbons obtained from the middle distillate, gas oil fraction during petroleum separation. Due to their massive production and use as fuels for transportation, they are among most common sources of organic pollutants for the surface soil. They also impact the subsurface soil through leaking from underground storage tanks and pipelines. Due to their mobility in the soil, such released diesel fuel hydrocarbon can reach water intakes or ground water reservoirs, thus generating relevant risks for human and other living organisms.(Wang x and Bartha.R,1990).

Besides the varying rates of biodegradation, researchers have consistently documented a lag time after oil is spilled before indigenous microbes begin to break down the oil molecules (Hoff, 1993). This lag time is related to the initial toxicity of the volatile fractions of the oil, which evaporate in the first few days of a spill. Microbial populations must begin to use oil and expand their population before measurable degradation takes place, a period usually lasting several days. This fact becomes very important when considering the appropriateness of bioremediation as a quick or first response technique (Hoff, 1993).

Biosurfactants (BS) are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension (ST) and interfacial tensions between individual molecules at the surface and interface, respectively. Bacteria make low molecular weight molecules biosurfactants such as glycolipids in which carbohydrates are attached to a long-chain aliphatic acid or lipopeptides. One of the best-studied glycolipids is rhamnolipid, produced by several species of ***Pseudomonas,*** which consists of two moles of rhamnose and

two moles of β-hydroxydecanoic acid (Lang S and Wullbrandt D, 1999). Recently, a new class of glycolipids, glucose lipids,produced by ***Alcanivorax borkumensis*** has been described (Abraham W R,1998 ;Golyshin P M et all.,1999 ;Yakimov MM et all.,1998). These consist of an anionicglucose lipid with a tetramericoxyacyl side chain.

There are at least two ways in which biosurfactants are involved in bioremediation: increasing the surface area of hydrophobic water-insoluble substrates and increasing the bioavailability of hydrophobic compounds.Due to increasing oil transportations the risk of large oil accidents is growing, it is important to research and develop ways to catch the oil from the environment in case something happens.

2. Materials and Methods

2.1 Isolation and identification of Oil degrading bacteria

Bushnell-Haas (BH) medium was used as the enrichment media with coconutoil, engine oil, diseal, supplied as the sole carbon source to isolate oil–degrading bacteria. 10 g of the contaminated soil, was added and incubated at 30°C at 170 rpm. After 1 week,1 ml of enriched media was transferred into freshly prepared enrichment media and incubated at the same conditions as described above. 1ml from the third enrichment process were plated out onto BH agar plates and incubated at 30°C. The single colonies were streaked onto nutrient agar plates, incubated at 30°C overnight. The oil-degrading isolates were identified by gram stain and biochemical tests

2.2 Determination of Biomass

The isolates were inoculated into 5 ml nutrient broth at 30°C overnight. The overnight culture were transferred into 100 ml BH medium with 5 ml (1%) used engine oil ,coconut oil, diesel and was incubated at 30°C at 160 rpm. A control devoid of the bacterial isolate was prepared for each set of experiments. The growth patterns were obtained by measuring the optical density at 580 nm .The dry weight technique was done to quantify microbial growth as bacterial density.

2.3 Biosurfactant activity tests

2.3.1 Oil displacement Test

Oil displacement is a method used to determine the diameter of the clear zone, which occurs after adding surfactant-containing solution on an oil-water interphase. The diameter evaluation allows the surface tension reduction efficiency of a given biosurfactant. In this test, 15 ml distilled water was added to a petri dish which is 90 mm in diameter. 100 µl of diesel was added to the water surface, followed by the addition of 20 µlof cell culture supernatant on to the oil surface. The diameter and the clear halo visualized under visible light was measured after 30 s

2.3.2 Emulsification capacity (E24)

A mixture of 2 ml hydrocarbon and 2 ml cell free extract obtained after the centrifugation of sample culture were taken in a test tube and homogenized by vortexing for 2 min. The emulsion activity was investigated after 24 hours and the emulsification index (E24) was calculated by the total height of the emulsion by the total height of the aqueous layer and then multiplying by 100.

2.3.3 Haemolytic activity

Haemolytic activity appears to be a good screening criterion for surfactant-producing strains. Isolated strains were screened on blood agar plates and incubated at 37°Cfor 24h. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carrillo et al., 1996).

2.3.4 Drop collapsing test

100 micro-liter of mineral oil was added to a narrow tube. Then the tubes were equilibrated for 1 h at room temperature, and then 250µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant-producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production (Youssef et al., 2004).

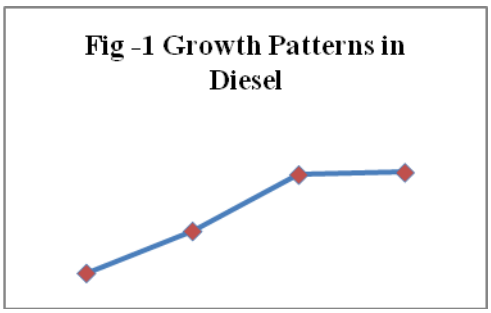
3. Results & Discussion

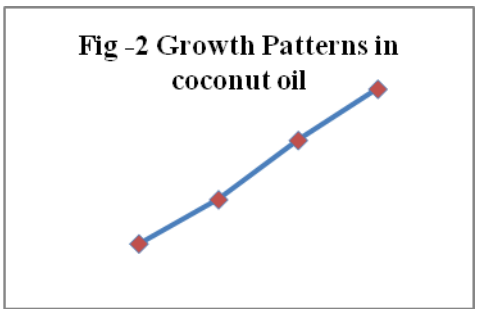
Two bacterial strains, capable of utilizing diesel as carbon source and one isolate was obtained from both engine oil and coconut oil as a carbon source were isolated from the contaminated soils. The isolates were identified as *Pseudomonas* Species, *Staphylococcus* Species, Non Lactose Fermenting *Proteus* using the biochemical tests and confirmed by using Selective media. *Pseudomonas sp(D1) and Proteus sp(D2) were isolated from Diesel. Pseudomonas sp(E) and Staphylococcus sp(C) were isolated from Engine oil and Coconut oil respectively* .On MacConkey agar, *Staphylococcus sp* formed as lactose fermenting pink colonies and on Mannitol salt agar, *Staphylococcus* sp showed pink colour. On Cetrimide agar, *Psuedomonas sp* appeared as bluish green colour. Non lactose fermenting ,gram negative species was supposed to be *Proteus sp.* Table 1 shows the preliminary tests conducted for identification of isolates .Biomass determination studies were observed for 72 hours. According to turbidity measurement studies the growth of microorganisms in diesel is rapidly increasing in two days .Growth of microorganisms in vegetable oil is increasing in the linear fashion. Growth of microorganisms in engine oil is slightly increasing, and the growth is rapid second day. Dry weight of *Non swarming Proteus and Pseudomonas sp* isolated from diesel *were* 47.36% and 31.8% respectively. *Pseudomonas sp* from engine oil showed dry weight 31.8% and *Staphylococcus sp* from coconut oil were of 30.7% dryweight.

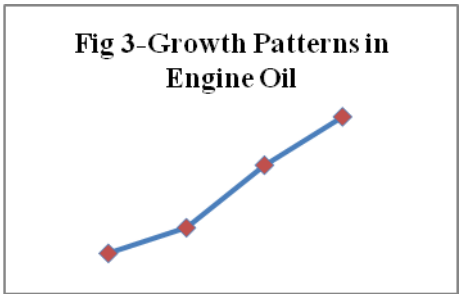
The positive result for Drop collapsing test was shown by *Pseudomonas species* in the engine oil in Fig -4. In Oil displacement test *Proteus sp* showed greater zone diameter ,thus it have high biosurfuctant property compared to others. *Staphylococcus sp* showed a high emulsification index (39.39cm) when compared to other organism. Haemolytic activity were shown by *Pseudomonas species* from engine oil and *Staphylococcus species* from coconut oil.

PRELIMINARY TESTS	***Pseudomonas* Species(E)**	***Pseudomonas* Species(D1)**	***Staphylococcus* Species(C)**	**Non Lactose Fermenting *Proteus(D2)***
Colony morphology	Too small,round,oily,appeared as creamy	Small,round,oily,opaque colonies	Irregular creamy ,small colonies	Small,round,oily ,opaque colonies
Gram staining	Gram negative	Gram negative	Gram positive	Gram negative
Motility	Motile	Motile	Non motile	Motile
Catalase test	Positive	Positive	Negative	Positive
Oxidase test	Positive	Positive	Positive	Positive

Table -1 Preliminary tests conducted for identification of isolates







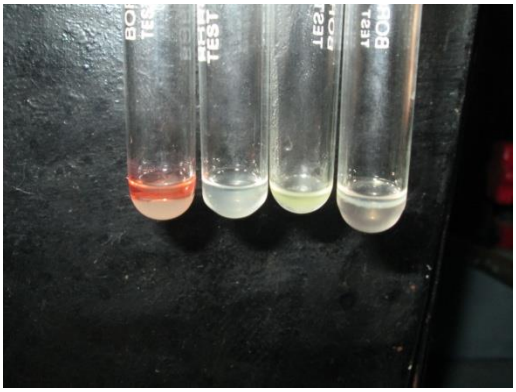


Fig -4 Drop collapsing test

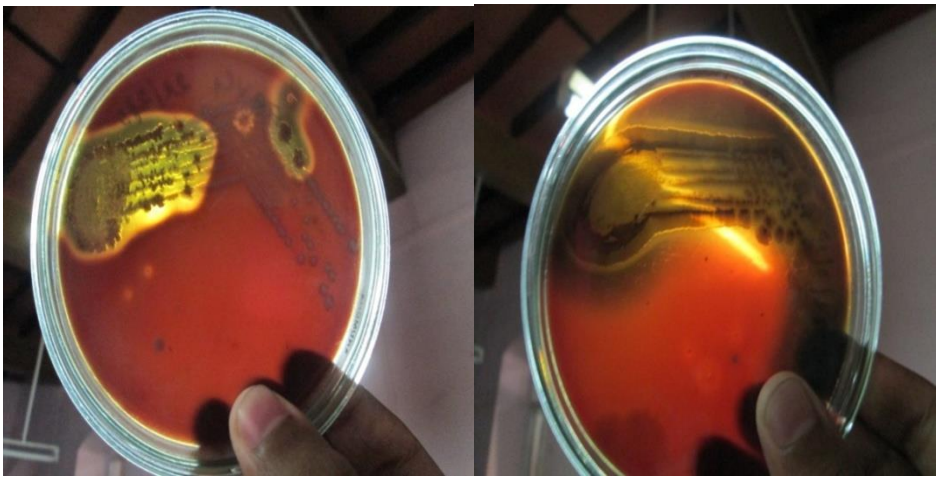


Fig -5 Haemolytic activity shown by *Pseudomonas species* and *Staphylococcus species*

4. Conclusions

Four bacterial isolates, *Pseudomonas sp (E) isolated from* Engine oil ,*Staphylococcus sp (C) isolated from* Coconut oil, *Pseudomonas sp (D1) and Proteus sp (D2) isolated from* Diesel and were obtained from oil–contaminated soil in this study. An increase in oil degradation was corresponding to an increase in cell number during the degradation processes demonstrating the ability of utilizing oil as the energy source. *Pseudomonas species* are the most common bacterial hydrocarbon–degraders reported in the literature . Biomass and dry weight rapidly increases in course of study. Study on biosurfactant properties were noted by performing oil displacement test, emulsification capacity (e24), haemolytic activity, drop collapsing test. Present study revealed that biological tools can reduce pollution of soil and water that occurred due to increased transport and industrialization, etc. The main advantages of microbiological method of bioremediation of hydrocarbon polluted sites are use of biosurfactant producing bacteria without necessarily characterization ofthe chemical structure of the surface active compounds. The cell free culture broth containing the biosurfactants can be applied directly or by diluting it appropriately to thecontaminated site. The other benefit of this approach is that the biosurfactants are very stable and effective in the culture medium (Płociniczak et al., 2011) that was used for their synthesis.

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Estimation of total sugar content in different banana cultivars of Kerala

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Abstract

India is the largest producer of Banana, the second largest produced fruit after citrus. Of the various cultivars of banana grown in Kerala, the sugar contents of five common cultivars were analyzed. Fruit obtained from the cultivar "palayamkodan" was richest in total sugar content per gram of the sample. This was closely followed by the variety " Poovan". The All the other varieties had a total sugar content lower than 20%. The total sugar content was the lowest in case of the cultivar named "Amrithavahini.

Key Words : banana, sugar content, cultivar, 'palayamkodan', 'Amrithvahiny'

1. Introduction

Banana is, perhaps, the most common fruit in the entire world. Banana is the second largest produced fruit after citrus, contributing 16% of the world's total fruit production. India is the largest producer of Banana, contributing to 27% of world banana production (Debabandya *et al.*, 2010). Plantain resemble banana but are longer in length, have a thicker skin, and contain more starch. They are also a major staple food in Africa, Latin America, and Asia. They are usually cooked and not eaten raw unless they are very ripe (IITA, 2012). However, in common usage, the term banana is used for both banana and plantain. The soft and sweet fruit of these tropical plants is enjoyed by people from around the world. There are hundreds (if not thousands) of variety of bananas found growing in different parts of the world.

In Kerala, there are about 50 cultivars of banana and all of them are different in taste. Edible bananas are derived from *Australimusa* and *Eumusa* series, which have different origins from same genus. Three common species of *musa* are widely grown in the world. *M. cavendishii, M. paradisiaca* and *M. sapientum*. *M.cavendishii* known as desert banana, is sweeter and less starchy than *M. paradisiaca*, while *M. sapientum*, known as true banana, is usually eaten raw when fully mature (Debabandya *et al.*, 2010).

The skin of the fruit is green when unripe and turns yellow when ripe. The skin is easily peeled off to reveal the edible flesh which, in most varieties, is white with tinge of yellow and is generally seedless. Starch is the main component of carbohydrate in unripe banana and as ripening progresses; it changes to soluble sugars (Robinson, 1999). The starch in green bananas slowly turns to sugar as the banana ripens. This enzymatic conversion of starch to sugar is responsible for the sweet and soft characteristics of bananas. While starch and sugar are chemically different molecules, this conversion does not affect the calorie content of a banana, only the perceived sweetness. Both starch and sugar are carbohydrates, providing four calories per gram. The types of sugar present in ripe bananas include glucose, fructose and

sucrose. Increased sweetness occurs quickly in the early stages of ripening and continues throughout the ripening process.

The breakdown of starch also produces fruit oligosaccharides, sometimes called maltodextrins. These are glucose chains with about nine glucose molecules linked together. Because these are produced as an intermediary of starch breakdown, oligosaccharides are sweeter than starch, but not as sweet as sugar.

Starch content in bananas drop from 25 percent to about 1 percent after fully ripe. Green bananas contain more of this resistant starch and may cause bloating and gas. Eventually, this resistant starch breaks down to sugars as bananas continue to ripen. The three natural types of sugars banana contain are sucrose, fructose & glucose. The sugar content in overripe bananas can be dramatically higher than in bananas that are under ripe and optimally ripe. They are also rich in different minerals (like potassium) and components like vitamin A, B_6 and C.

As the sugar content in banana increases, the perceived sweetness of the fruit also increases. This increases its palatability. Studies on sugar content in banana varieties have been carried out in various parts of the world. A study by Marriott *et. al.*, (1981), found that total sugar content was 23% in fully ripe bananas and 20% in plantains. The ratio of glucose:fructose was approximately unity for bananas and plantains at all stages Sucrose comprised more than 70% of the total sugars in fully ripe bananas and plantains.

A review of literature showed that the reported sugar content in Bananas varied widely. Henderson *et al.*, (1959) observed that percentage of reducing and non reducing sugar in *Musa cavendishii* was 8.4 & 8.9% in samples collected from New South Wales, Australia. Debabandya *et al.*, (2010) reported that total sugar content from banana varieties in India was as high as 40% at ripeness. On the other hand, a study by Alagarsamy Ramesh Kumar & Neelakandan Kumar found that total sugar content in bananas from Tamil Nadu varied from 14 to 19 % (2008).

However, studies on sugar content in common cultivars of kerala are not frequent. On such study was carried out by Asha Latha (2001) who analyzed the sugar content of three varieties of banana namely red banana, poovan and nendran. She observed that the total carbohydrate content in red banana was low. The study estimated the reducing sugar content in these varieties and found that it was high in poovan (rasthali). We did not come across any study that estimated the total sugar content in different varieties commonly produced and consumed in Kerala.

Of the various cultivars of banana commonly grown & consumed in Kerala, five were selected for the study. Brief descriptions of these are given below. The names of cultivars are given as in the local language - Malayalam.

'Ethan or nenthran' – It is a popular variety of banana in Kerala where it is relished as a fruit, used in a variety of culinary preparations, as well as used for processing . Fruits have a distinct neck with thick green skin turning buff yellow on ripening. Fruits remain starchy even on ripening. The fruit is a bit harder than many other varieties of bananas.

'Poovan'– It is a leading commercial cultivar grown throughout the country. Fruit is slightly acidic, firm and has typical sour-sweet aroma. Fruits turn to attractive golden yellow on ripening. This banana fruit variety is fairly larger in size than kannaan, palayam kodan etc, is bright glossy yellow in appearance. Prices are high for this variety.

'Njaly poovan'- In Kerala it was backyard cultivar, however due to increasing demand it is now cultivated on a large scale. Fruits are small and pulp is ivory white in colour. Fruit is highly fragrant, tasty, powdery and firm.

'Amritha vahini' comaparatively small in size, slender with a prominent beak and are packed closely around the axis having a windblown appearance.

'Palayam kodan' – This is the most widely cultivated variety of banana in Kerala, very soft when fully ripe.

2. Materials and Methods

The total sugar content in the fruit was estimated by Dubois *et al.*, method (1956), as it is rapid, reproducible and accurate. The phenol-sulphuric acid spectrophotometric method (Dubois *et al.*, 1956) was used for the determination of sugar content in bananas. Standard glucose solution of 0.01% was first prepared which was then serially diluted to prepare standard solutions of concentration ranging from 5-50ppm.

1ml of each solution of different concentration was pipetted into a test tube and 1ml of 5% phenol was added and mixed gently. Five ml of concentrated sulphuric acid and nine ml distilled water were added to bring the volume to 20 ml. It was allowed to stand for 10 min, shaken well and immersed in a water bath at 30^0 C for 20 min. A yellow orange colour developed and the absorbance was read at 490nm using 4049 spectrophotometer. The formation of colour is due to the interaction of phenol and sulphuric acid with sugar. The intensity of the color is a function of the amount of phenol added. Sugar undergo dehydration in the presence of suphuric acid to furfural or hydroxyl methyl furfural that condense with phenol to form yellowish orange compound with an absorption maxima at 490nm. The absorbance was read at 490nm using UV mini 1240 spectrophotometer.

2.1 Extraction of sugar

Different types of ripe bananas were used as raw material for this study. All the bananas selected were of optimum ripeness without blemishes. The bananas were peeled and a piece of about 2gm was weighted accurately. A mortar and pistil was washed well with distilled water and dried. The banana piece was mashed well and a small amount of distilled water, around 10ml, was added to it. Extraction was done by heating the sample to boiling for two minutes and stirring occasionally. The extract was filtered into a 100ml standard flask and made up to the mark. From this solution, 1ml was pipetted out in to a 100ml standard flask and again made up to the mark.. The solution so obtained is expected to be well within the concentration range of standard sugar solutions prepared.

One ml of this solution was pipetted out and to this 5% phenol was added and mixed gently, before five ml of conc. sulphuric acid and nine ml distilled water were added to bring the volume to 20 ml. It was allowed to stand for 10 min, shaken well and immersed in a water bath at 30^0 C for 20 min. A yellow orange colour developed and the absorbance was read at 490nm using UV mini 1240 spectrophotometer.. The absorbance so obtained was recorded and from this value the concentration of test solution determined by graphical method

2.2 Extraction of sugar

All the bananas selected were of optimum ripeness without blemishes. The bananas were peeled and a piece of about 2gm was weighted accurately. A mortar and pistil was washed well with distilled water and dried. The banana piece was mashed well and a small amount of distilled water, around 10ml, was added to it. Extraction was done by heating the sample to boiling for two minutes and stirring occasionally. The extract was filtered into a 100ml standard flask and made up to the mark. From this solution, 1ml was pipetted out in to a 100ml standard flask and again made up to the mark. The solution so obtained is expected to be well within the concentration range of standard sugar solutions prepared.

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3.0 Results & Discussion

The weight of sample, absorbance value and the total sugar content in different cultivars are given in table 1.

Table 1.

Sl. No.	Sample name	Absorbance	Total sugar in test sample (ppm)	Total sugar content in sample (%)
1	Palayamkodan	0 .070	52.5	26.25
2	Poovan	0.063	45	22.5
3	Njalipoovan	0 .052	33	16.5
4	Nenthran	0.048	29	14.5
5	Amrithavahini	0 .044	24	12

It was observed that fruit obtained from the cultivar “palayamkodan” was richest in total sugar content per gram of the sample. The total sugar content in this variety was around 26.25%. This was closely followed by the variety “ Poovan”. The total sugar content in this case was 22.5%. All the other varieties had a total sugar content lower than 20%. The total sugar content in “Njalipoovan” and “Nenthran” were 16.5 and 14.5% respectively. The total sugar content was the lowest in case of the cultivar named “Amrithavahini. The values are given below

4. Conclusions

The study led to the conclusion that total sugar content in common banana cultivars of kerala vary in their total sugar content. Of the various cultivars analysed the total sugar content was highest in "palayamkodan" and lowest in "Amrithavahini".

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Study on Quality Analysis of selected commercial milk samples

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Abstract

Milk, a translucent white liquid produced by the mammary glands of mammals which acts as the primary source of nutrition for young mammals contains all the food stuffs in an easily assimilable form and it is recommended to young and old people, being considered a complete food. The high nutritive value makes milk an ideal medium for the rapid multiplication of bacteria, particularly under unhygienic production and storage at ambient temperatures which may cause serious health problems when consumed. Another major consideration while ensuring the quality of milk is adulteration, which is the addition of any material to the milk, or removal of any constituent of milk. In the present study, an attempt was made to analyse some common quality control parameters of five market samples of pasteurized milk. The milk samples were subjected to physico-chemical, microbiological and adulterant detection tests. The analyses were performed using random, well mixed samples and the results were compared to ascertain the quality of different brands of commercially available milk samples.

Keywords: Milk, MBRT, Coliforms, Adulterants, Preservatives

1. Introduction

Milk is a translucent white liquid produced by the mammary glands of mammals which acts as the primary source of nutrition for young mammals (William & Lawrence, 2005) and contains all the food stuffs in an easily assimilable form. The role of milk in nature is to nourish and provide immunological protection for the mammalian young and it is recommended to young and old people, being considered a complete food. One kilogram of milk contains: water 84-90%, fat 2-6 %, protein 3-4 %, lactose 4-5 %, minerals < 1 % and supplies about 668 Kcal (Agatha & Elena, 2009). Though the original adaptive significance of milk secretions may have been nutrition or immunological protection (Lefevre *et al.*, 2010; Vorbach *et al.*, 2006), it has acquired a more complex and elaborate status over time.

The high nutritive value makes milk an ideal medium for the rapid multiplication of bacteria, particularly under unhygienic production and storage at ambient temperatures. Milk which leaves the udder of the animals is sterile but can be a good source for microorganisms to grow and multiply, if contaminated during the course of handling and processing. Presence of bacteria like *Escherichia coli* is of considerable public health significance, as it indicates faecal contamination. A variety of micro organisms such as *Shigella, Salmonella, Yersinia, Klebsiella, Pseudomonas, Vibrio, Brucella, Listeria, Clostridium, Mycobacterium, Coxiella* (heat resistant; index organism for pasteurization) may cause different types of food borne illnesses, food intoxication and food infections (Boor, 2001; Rowan, 2001). Another major consideration while ensuring the quality of milk is its adulteration, which is the addition of any material to the milk, or removal of any constituent of milk. Milk quality control is the use of approved tests to ensure the application of approved practices, standards and regulations concerning the milk and milk products. The tests are designed to ensure that milk products meet accepted standards for chemical composition and purity as well as levels of different micro organisms (Marshall, 1992).

In the present study, an attempt was made to analyse some common quality control parameters of five market samples of pasteurized milk. The study included physico-chemical parameters like pH, Clot on Boiling, Microbiological Tests like MBRT (Methylene Blue Reduction Test), presence of coli forms and tests for the presence of adulterants and preservatives. The analyses were performed using random, well mixed samples and the results were compared to ascertain the quality of different brands of commercially available milk samples.

2. Materials and Methods

2.1. Milk Samples

Commercial pasteurised samples of milk were purchased from local market and subjected to various analyses to examine their quality. The five samples tested were of the brands Aroma (S_1), Cowma (S_2), Milma (S_3), Milway (S_4) and Shakti (S_5). Microbiological analyses were performed under aseptic conditions. Raw milk obtained fresh was used as control for the tests.

2.2. Analytical Parameters

2.2.1. pH of Milk using pH paper

This test determines the freshness of milk. pH paper strips (HiMedia, Mumbai) of the pH range are dipped in a small quantity of milk and compared the colour changes with standard chart to note the pH.

2.2.2. Clot on Boiling Test (COB)

It is a quick and simple test to determine the keeping quality of milk. About 5ml of well mixed milk was boiled in a water bath for 5 min. to check for the keeping quality of milk. The clotting of milk on the side of tube or at the bottom showed that milk is not good.

2.2.3. Methylene Blue Reduction Test (MBRT)

MBRT is one of the most important tests for assessing the shelf life of milk. It is also useful in checking whether milk is properly pasteurized or not. About 30 ml milk was taken in a screw capped boiling tube and 3 ml of methylene blue dye mixed with it under aseptic conditions and incubated in a water bath at a temperature of 37ºC. The colour was noted every 30 min. till the milk was completely decolourised. The samples were inverted and mixed in between to ensure there is complete uniformity. The dye which was initially in the oxidised form was reduced to white by the bacteria present in milk microorganisms and the time taken for it was inversely proportional to the number of bacteria present in the milk.

2.2.4. Detection of Coli forms

The presence of coli forms (*Escherichia coli*) is an indication of contamination of milk with fecal matter, either through water, dirty utensils, unclean hands of workers etc. The milk samples were streak plated onto Eosin Methylene Blue (EMB) agar to check for the presence of small dark centred colonies with metallic sheen which indicated the presence of *E. coli.*

2.2.5. Tests for the presence of Adulterants

2.2.5.1. Detection of Cane sugar

Sugar is added to increase the density or the percentage solids content of milk that is diluted with water and thus to prevent detection of added water. About 10ml of milk was taken in a test tube and mixed with 1ml of conc. HCL followed by the addition of 0.1g of resorcinol powder. It was mixed thoroughly and incubated in a boiling water bath for 5 min. The colour formed was noted. Red colour indicated presence of added cane sugar.

2.2.5.2. Detection of Starch

Addition of starch increases the SNF content of milk. Wheat flour, rice flour, etc., can also be added for increasing the SNF content. To detect the presence of added starch, few drops of 1% iodine solution was added to thoroughly mixed sample of milk and observed for the development of blue colour, which shows that starch is present.

2.2.5.3. Detection of added Urea

Urea is generally added in the preparation of synthetic milk to raise the SNF value. Two ml of milk was taken in a test tube and mixed with 2ml of amino benzaldehyde and observed for colouration. Presence of deep yellow colour indicated the presence urea in milk while a light yellow colour indicated the natural urea content of normal milk.

2.2.5.4. Detection of Glucose

Poor quality glucose is sometimes added to milk to increase the lactometer reading. To about 3 ml of milk taken in a test tube 3 ml Barford's reagent was added and mixed thoroughly. It was further kept in a boiling water bath for 3 min and then cooled for 2 min. by followed by the addition of 1 ml of phosphomolybdic acid and shaken well. Development of blue colour showed that glucose was present in the milk sample.

2.2.5.5. Detection of Salt

The density of milk which is diluted with water can be increased by the addition of salt. About 5 ml of silver nitrate reagent was taken in a test tube and mixed with 2-3 drops of potassium dichromate reagent and 1 ml of milk was added to it. The appearance of yellow colouration indicated that the sample contained salt. A chocolate or reddish brown colour showed that the milk sample was free from salt.

2.2.5.6. Detection of Soap

10 ml of milk taken in a test tube was diluted with equal quantity of hot water followed by the addition of 1 – 2 drops of phenolphthalein indicator. Development of pink colour indicated that the milk is adulterated with soap.

2.2.5.7. Detection of Skim Milk Powder

The milk sample is mixed with few drops of nitric acid and the colour is noted to check for the adulteration with skim milk powder. Samples with skim milk powder showed orange colour while unadulterated ones were of yellow colour.

2.2.6. Tests for the presence of Preservatives

2.2.6.1. Detection of Formalin

Formalin (40%) although poisonous, can preserve milk for a long time. 10 ml of milk was taken in a test tube and 5 ml conc. sulphuric acid added through the sides of the test tube without shaking. A violet or blue ring at the intersection of the two layers, showed the presence of formalin.

2.2.6.2. Detection of benzoic and salicylic acid

To about 5 ml of milk taken in a test tube, 3-4 drops of concentrated sulphuric acid and 0.5% ferric chloride solution were added drop by drop and mixed well. Development of buff colour indicated presence of benzoic acid and violet colour indicated presence of salicylic acid.

3. Results and Discussion

The milk samples were subjected to physico-chemical, microbiological and adulterant detection tests. The samples were thoroughly mixed to disperse the constituents before the tests. Bacteriological tests were performed using sterile glass wares under aseptic conditions. The results obtained are discussed below.

3.1 Physico-Chemical Tests

3.1.1 pH of Milk

pH paper strips incorporate certain indicators which show change in colour with the change in pH. The pH paper strips impregnated with these indicators are available in narrow range and wide range. They can be efficiently employed to obtain primary information about the freshness of milk since normal milk pH is between 6.6-6.8. pH above 6.9 is indication of mastitic milk/late lactation milk. Among the tested samples, all were of the range 6.5-6.9 and were considered to be normal.

3.1.2 Clot on Boiling Test (COB)

COB is one of the old tests for too acid milk (pH<5.8) or abnormal milk (e.g. colostral or mastitis milk). For good quality of milk COB should be ¯ve i.e. no clot should form on boiling. Clotting of milk on the side of tube or at the bottom indicates poor keeping quality. COB^{+} ve milk should be separated and not mixed with good milk. Among the samples, S_2 and S_3 developed solid clots on boiling while S_1, S_4, S_5 and raw milk were COB ¯ve. Thus S_2 and S_3 were found to fail the test and this could be due to acid or rennet producing microorganisms or an abnormal high percentage of proteins like colostral milk. Such milk cannot withstand the heat treatment in milk processing and is better rejected.

3.1.3 Methylene Blue Reduction Test (MBRT)

Dye reduction tests are indirect method of estimation of total bacterial content of milk by making a correlation between the time required to reduce dyes to colourless in milk. Generally the time required for reduction of dye is inversely proportional to the number of bacteria present in the milk. The milk will have dissolved oxygen content and so initially it is in oxidized state and when the bacteria multiply utilizes the oxygen and after some time total

oxygen content in milk is exhausted and the milk is in reduced stage. The dyes used will have colour in oxidized stage and become colourless in reduction stage.

In the present study, S_1 sample could resist decolouration till 6 hrs and was found to be of superior quality than the other samples. The milk samples were graded as follows

Sample	Time required for Reduction (hrs)	Grade/ Quality of milk
S_1	5 and above	Very Good
S_2, S_4, S_5	1 and 2	Fair
S_3	0.5 and below	Poor

Table 1. MBRT grading of milk samples

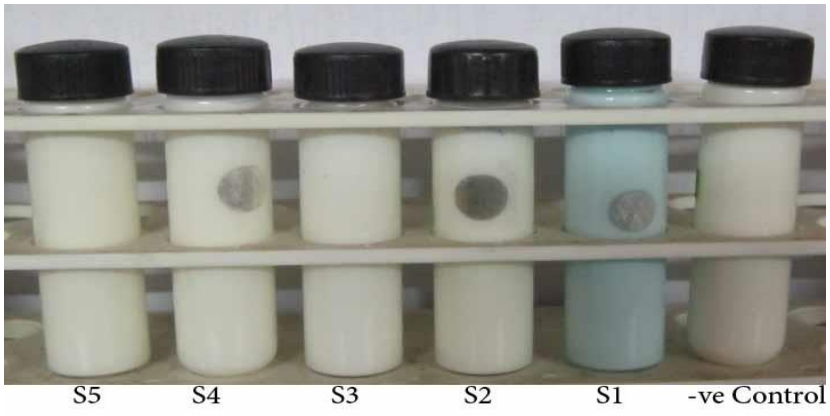

Figure 1 : MBRT test of Milk samples

3.1.4 Detection of Coliforms

The milk samples were plated onto EMB agar to check for the presence of *E. coli*, which indicates faecal contamination. Typical colonies of coli form organisms will appear pink with dark centre and metallic sheen on EMB agar. Raw milk sample was also plated as a control. After 24 hr incubation, distinctive small, dark centred colonies with metallic sheen could be observed in all the samples except S_1 and raw milk. There were a few colonies from S_2, S_3, S_4 and S_5.

Presence of *E. coli* is of significance since it could indicate the unhealthy reception and handling of milk samples or the possibility that it is contaminated with dirty water. This further shows that pasteurization does not yield sterile product and boiling of milk before consumption is mandatory. It is also important that pasteurized products be consumed within the stipulated time period and not stored for long.

3.1.5 Tests for the presence of Adulterants and Preservatives

The common adulteration of milk is addition of water which results in the dilution of milk constituents (fat and solid non fat -SNF). The water adulterated milk will be thin and gives less lactometer reading due to less SNF content. To overcome this, other compounds such as cane sugar, starch/cereal flour, skim milk powder, gelatine, urea, ammonium sulphate and glucose are added so that milk shows required lactometer reading. Fat being the costly ingredient of milk, some portion of fat is removed.

Removal of fat also comes under adulteration of milk. Buffalo milk is richer than cow milk in almost all the constituents. Hence watered buffalo milk is used as an adulterant of cow milk.

Micro organisms are susceptible to the action of chemicals known as preservatives, antiseptics, disinfectants, germicides etc which either check their growth or destroy the organisms and then keep the milk for a longer time. Addition of chemicals is illegal and therefore, common methods have been developed to detect their addition. The milk samples were tested for the presence of common adulterants like cane sugar, starch, urea, glucose, salt, soap, skim milk powder and preservatives such as formalin, benzoic acid, salicylic acid etc.

The samples S_2, S_3, S_4, showed the presence of cane sugar and glucose while S_5 and S_1 were completely negative for all the adulterants tested. The results were tabulated as follows.

Sample	**Adulterants**						
	Cane sugar	Starch	Urea	Glucose	Salt	Soap	Skim milk powder
S_1	-- No Red colour	-- No Blue colour	-- No Yellow colour	-- No Blue colour	-- No Chocolate colour	-- No Pink colour	-- No Orange colour
S_2	+ Red colour	-- No Blue colour	-- Faint Yellow colour	+ Blue colour	-- No Chocolate colour	-- No Pink colour	-- No Orange colour
S_3	+ Red colour	-- No Blue colour	-- Faint Yellow colour	+ Blue colour	-- No Chocolate colour	-- No Pink colour	-- No Orange colour
S_4	+ Red colour	-- No Blue colour	-- Faint Yellow colour	+ Faint Blue colour	-- No Chocolate colour	-- No Pink colour	-- No Orange colour
S_5	-- No Red colour	-- No Blue colour	-- Faint Yellow colour	-- No Blue colour	-- No Chocolate colour	-- No Pink colour	-- No Orange colour
Raw Milk	-- No Red colour	-- No Blue colour	-- Faint Yellow colour	-- No Blue colour	-- No Chocolate colour	-- No Pink colour	-- No Orange colour

Table 2. Presence of adulterants in milk samples

Sample	Formalin	Benzoic & Salicylic acid
S_1	-- No violet or blue ring	-- No Buff or violet colour
S_2	-- No violet or blue ring	-- No Buff or violet colour
S_3	-- No violet or blue ring	-- No Buff or violet colour
S_4	-- No violet or blue ring	-- No Buff or violet colour
S_5	-- No violet or blue ring	-- No Buff or violet colour
Raw Milk	-- No violet or blue ring	-- No Buff or violet colour

Table 3. Presence of preservatives in milk samples

All the samples S_1, S_2, S_3, S_4, and S_5 were negative for the preservatives tested.

4. Conclusions

Thus, in the present study, five commercial pasteurised samples of milk of the brands Aroma (S_1), Cowma (S_2), Milma (S_3), Milway (S_4) and Shakti (S_5) procured from the local market were subjected to various physico-chemical, microbiological and adulterant detection tests to examine their quality. The study concluded that

- S_3 failed Clot on Boiling test while S_1, S_4, S_5 were COB ¯ve.
- S_1 could resist decolouration till 6 hrs in MBRT test while others were either of poor or fair grade.
- S_1 showed no distinctive colonies of *E. coli* while S_2, S_3, S_4 and S_5, showed the presence of the same**.**
- S_2, S_3, S_4, showed the presence of cane sugar and glucose while S_5 and S_1 were completely negative for all the adulterants tested.
- S_1, S_2, S_3, S_4, and S_5 did not contain any of the preservatives tested.

Among the samples tested, S_1 of the brand Aroma was found to be of good quality.

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Solubility behaviour of aqueous solutions of some aromatic carboxylic acids in presence of inorganic salts

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Abstract

Solubility is an important parameter in Chemistry. To draw a correlation between the solubility behavior of organic and inorganic compounds is always interesting. The current work consists of studying variations in the solubility of a few aromatic carboxylic acids like benzoic acid, salicylic acid and cinnamic acid in presence of inorganic salts. Salting out phenomenon was directly linked to the concentration of the electrolyte. The study is helpful to understand how to separate ions in solution by selective precipitation.

Keywords: Solubility, inorganic salt, Benzoic acid, Salicylic acid and Cinnamic acid

1. Introduction

Solubility is of fundamental importance in a large number of scientific disciplines and practical applications, ranging from ore processing, to the use of medicines (Avdeef, 2007) and the transport of pollutants. Organic compounds are normally less soluble in aqueous solutions. The solubility of an organic compound in aqueous phase is of importance in nature particularly in the case of sea water (Saylor et al, 1952)

An organic acid is a compound that shows the properties of the carboxylic acid group and containing carbon chain, as do all the organic compounds. The most common acids are the carboxylic acids whose acidity is associated with the carboxylic group –COOH. The acidity of an acid is determined by the relative stability of the conjugate base of the acid. This acidity is however usually weak and is the result of the –OH, -SH and the phenol group. Factors such as temperature (Gordon and Thorne, 1967) and pressure will alter this balance, thus changing the solubility (Mortimer, 2008).

The main objective of the current study is to determine the solubility of aromatic carboxylic acids in aqueous phase and to study the salting out phenomenon of aromatic carboxylic acids in presence of inorganic salts. These experiments help to investigate the application of the principles of solubility. In the current work the variation in solubility of salts like NaCl, KCl, $MgCl_2$ in saturated solutions of various organic acids is studied.

2. Materials and methods

Aromatic carboxylic acids like Benzoic acid, Salicylic acid and Cinnamic acid, inorganic salts KCl, NaCl and $MgCl_2$, phenolphthalein, NaOH and distilled water are used after purification if necessary.

The theory behind current experiments is that on addition of an electrolyte to a saturated solution of a weak acid, some of the dissolved substance gets separated *ie*, the solubility of the

substance decreased with the addition of the electrolyte. The detailed procedure (Yadav, 2008; Khosla et al., 1982) of solubility studies of carboxylic acids are given below:

First part of the experiment is the standardization of NaOH and the second part is the preparation of saturated solution and the determination of solubility.

V cm^3 be the volume of 0.05 M NaOH used to neutralize ***a*** g of the saturated carboxylic acid solution with or without salt. The weight of the acid (say b) present in the solution is given by

$$b = \frac{0.05 \times MW \times V}{1000}$$

The solubility, S of the acid can then be calculated from

$$S = \frac{b \times 1000}{(a-b) \times 122.1}\ moles/1000g$$

3. Results and discussion

3.1 Solubility of carboxylic acids under study

Solubility of each carboxylic acid was determined. For this a saturated solution of the acid is prepared in water. Their solubility obtained is given in figure 1 and table 1.

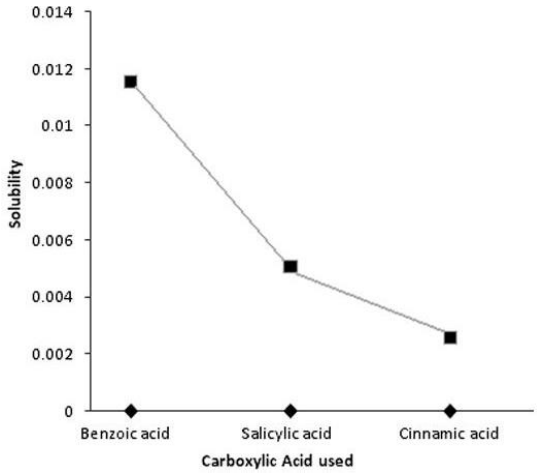

Figure 1: Solubility plot of carboxylic acids

Acid used	Solubility
Benzoic acid	0.01152
Salicylic acid	0.00505
Cinnamic acid	0.00258

Table 1: Solubility (in moles/1000g) of carboxylic acids

It is observed that the solubility of Benzoic acid > Salicylic acid > Cinnamic acid. The very low solubility of these carboxylic acids is due to the presence of aromatic benzene ring in their structure which is non-polar and prefers non polar solvents. The partial solubility of these acids in water is due to the presence of carboxyl functional group, which can form hydrogen bonds with water molecules.

3.2 Variation in solubility of carboxylic acids in presence of inorganic salts

The objective of the current study is to study the change in solubility of aromatic carboxylic acids in presence of various inorganic salts. The inorganic salts used were chlorides of sodium, potassium and magnesium. As expected, it is observed that in the case of each acid the solubility decreases as the salt content increases. Table 2 gives the variations in solubility of carboxylic acids in presence of salts. Careful analysis of the tables shows that the rate of decrease of solubility is highest for benzoic acid, *ie*, when the percentage of salt increase from 2 % to 10 %, there is a 10-20 % decrease in solubility is observed. But in the case of salicylic acid and cinnamic acid, the increase in salt content decreases the solubility of carboxylic acid to a lesser extend. In these cases when percentage of salt increases from 2-10 %, there is a 3-5 % decrease in solubility is observed.

3.3 Comparison in solubility of carboxylic acids in presence of inorganic salts

In the current study it is possible to compare the effect of inorganic salt on a particular acid. In the case of salicylic acid and benzoic acid, highest solubility is observed when the added salt is potassium chloride. The solubility of cinnamic acid is comparable in NaCl and $MgCl_2$**.** The variation in the solubility of carboxylic acids in presence of inorganic salts is given in tables 5-8. In all the cases, as concentration of the electrolyte increases, solubility decreases. The current work cannot correlate the nature of the salt and changes in solubility. The study is helpful to understand how to separate ions in solution by selective precipitation.

Acid used	**% of salt**	**Solubility** *(in moles/1000g)*		
		NaCl	**KCl**	**$MgCl_2$**
Salicylic acid	**2**	0.00458	0.00469	0.00422
	4	0.00436	0.00425	0.00378
	6	0.00425	0.00414	0.00356
	8	0.00382	0.00403	0.00334
	10	0.00348	0.00382	0.00313
Benzoic acid	**2**	0.00824	0.00988	0.00926
	4	0.00637	0.00787	0.00875
	6	0.00561	0.00650	0.00783
	8	0.00512	0.00613	0.00750
	10	0.00413	0.00525	0.00725
Cinnamic acid	**2**	0.00228	0.00167	0.00228
	4	0.00182	0.00121	0.00197
	6	0.00137	0.00759	0.00151
	8	0.00759	0.00606	0.00137
	10	0.00455	0.00455	0.00121

Table 2: Comparison of solubility trends of carboxylic acids in presence of different salts

4. Conclusion

The current work consists of studying variations in the solubility of a few aromatic carboxylic acids like benzoic acid, salicylic acid and cinnamic acid in presence of inorganic salts. As the concentration of the electrolyte increases, the salting out of acid was found to be increasing. A compilation of solubility data is presented and interpreted showing that solubility can be adequately correlated for a given electrolyte as a function of the organic solute's molar volume. The solubility changes observed due to difference in used electrolytes are not correlated, for which theoretical treatments are required. The variations in the solubility of carboxylic acids in presence of inorganic salts indicate that as concentration of the electrolyte increases, solubility decreases. The study is helpful to understand how to separate ions in solution by selective precipitation. As an extension to this work it is possible to carryout the solubility studies as a function of temperature.

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Characterization of major flavanoid pigment Anthocyanin from Brassica oleracea var. capitata f. rubra and role in DNA binding during infections.

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ABSTRACT

Anthocyanin a flavonoid pigment seen in most of the higher plants has been under intensive study from the past three decades. Anthocyanin pathway genes have been seen to have acquired lately in the evolutionary diversification of flavonoid In this paper anthocyanin extracted from red cabbage, was studied on its nature of changing colour accordingly to pH, which was basically due to change of charge, its antimicrobial properties, reducing power, as UV protectant/ sunscreen and ability to bind DNA during the process of pathogenesis, majorly due to fungal infection. Thus it has been assumed that fungal infection elicits and augments the production of anthocyanin. Here we have tried to understand, whether the existing anthocyanin pigment within the plant cell interacts with the genome, to increase the rate of anthocyanin expression in vivo.

Key words: Anthocyanin, Flavonoids, Brassica oleracea var. capitata f. rubra (red cabbage), EMSA.

1. Introduction:

Red cabbage (*Brassica oleracea var. capitata f. rubra)* is the red or burgundy-coloured cabbage, which are a common native crop in the Mediterranean region of Europe and now grows all over the world. Around the beginning of 19th century, investigators knew the colouring matter in red cabbage and certain other plants would turn different colours depending on the acidity or alkalinity of the solution. By the 20th century even a rough idea of molecular structure was elucidated .The red cabbage contains several types of anthocyanin having different substituents and functional groups, mostly in the form of glucosides. But they are easy to isolate as the functional groups are not tightly bound. Nearly 23 types of anthocyanin are found in red cabbage. A pigment is a material that changes the colour of reflected or transmitted light as the result of wavelength selective absorption. Many biological structures, contains pigments which is used for many biological purposes like camouflage, mimicry, sexual selection and attraction, photosynthesis, signaling, protection from radiations and sunburn. Anthocyanin is a water soluble pigment seen in higher terrestrial plants like bromeliads, berries, red cabbage, many fruits, flowers, carnivorous plants, providing colours ranging from shades of red through crimson, blue/purple, yellow and even colourless and can be classified as both flavonoid and phenolic pigment.

Anthocyanins apparently play a major role in very different plant processes; one for, attracting insects for the purpose of pollination as it absorbs ultra violet rays strongly (Harborne & Williams, 2000). The second major role, anthocyanin-related pigments serve as a UV screen, protecting plants DNA from damage which are produced in response to exposure. And in third, and no less significant role, anthocyanins serve as antifecdents, their disagreeable taste serving to deter predatory animals. Anthocyanins pigments can be produced by growing plant cells in tissue culture. It has been found invitro that when induced by any fungal infection, plant cells starts to produce anthocyanin in a higher rate.

Anthocyanin is also a valuable colourant in the food industry. Foods rich in anthocyanin is found to offer protection against cardiovascular diseases, certain kinds of cancer and some chronic diseases (Hou et al., 2003) (Murillo & Mehta 2001; Keck & Finley, 2004). These properties are because of their strong antioxidant activity. Even the complete anthocyanin biosynthesis has been studied extensively (Grotewold, 2006, Harborme & Williams, 2000).

Many environmental factors affects anthocyanin production, which includes light (intensity and wavelength, with blue and UV being most effective), temperature, water and carbohydrate level, concentration of the elements nitrogen, phosphorous and boron in the growth medium. Anthocyanin pigments are assembled from two different streams of chemical raw material in the cell: both starting from the Co_2 unit acetate derived from photosynthesis, one stream involves the shikimic acid pathway to produce the amino acid Phenylalanine. The other stream produces 3 molecules of Malonyl-coenzyme A, a C_3 unit. The streams meet and are coupled together by the enzyme chalcone synthase (CHS), Which forms an intermediate chalcone via a Polyketied Folding mechanism that is commonly found in plants. The chalcone is subsequently isomerized by the enzyme Chalcone isomerase (CHI) to the prototype pigment Naringenin, which is subsequently oxidized by enzymes like Flavonoid hydroxylase and coupled to sugar molecules by enzymes like UDP-O-glucosyl tranferase to yield the final anthocyanins. More than five enzymes are thus required to synthesize these pigments, each working in concert. Any even minor disruption in any of the mechanism of these enzymes by either genetic or environmental factors would halt anthocyanin production.

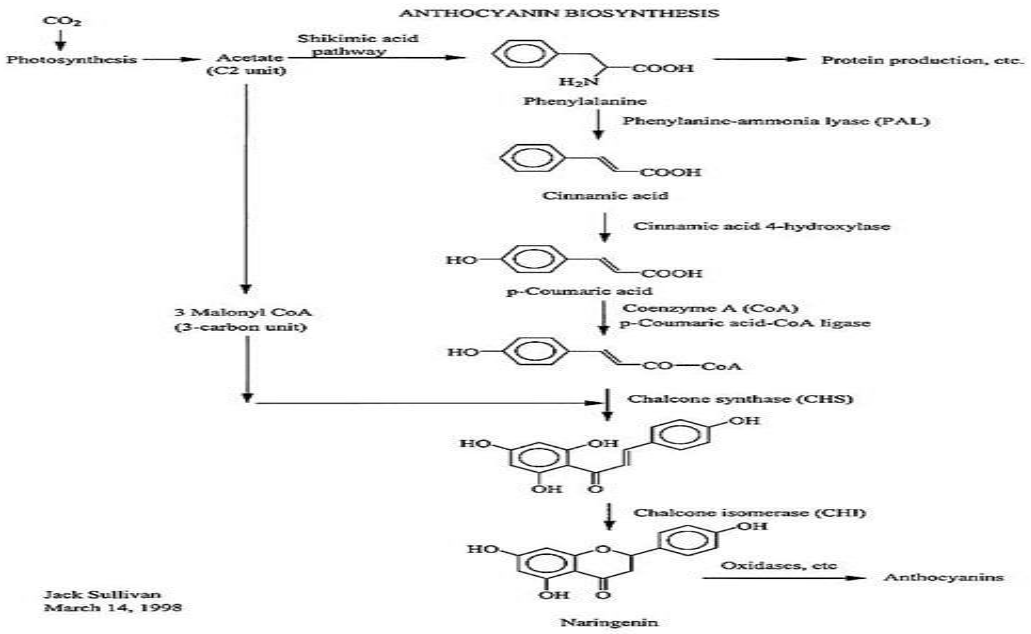

Figure 1: Anthocyanin biosynthesis pathway

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It has been found that fungal elicited cell suspension stimulated production of anthocyanin. It has revealed an antagonism between flavonoid/ naphtodianthrone and anthocyanin pathways (L. Rajendran et al., 1994, Sonja Gadzovska et al., 2012). The plants have evolved strategies to counteract herbivore attacks (Bennett & Wallsgrove 1994; Harborne, 1997). Therefore, a complete understanding of the properties of the pigment anthocyanin is biosynthesis and regulation is important to develop anthocyanin-rich foods to meet the increasing demand for health-promoting components in our diet.

Here the aim was to extract anthocyanin, check its purity, concentration, absorption spectrum, determining its antimicrobial activity and reducing power and to understand the interaction between anthocyanin pigment and cabbage DNA using EMSA.

2 Materials and methods

2.1 Plant source

The burgundy-red cabbage obtained from the departmental stores.

2.2 Microbial strains

We used the wild varieties of bacterial (*E.coli, Bacillus subtilis, Staphylococcus, Streptococcus, Pseudomonas, Salmonella)* and fungal strains (*Aspergillus niger, Fusarium, Alternaria, Rhizopus*) obtained from POLYCLINIC, Thrissur, for antimicrobial activity tests.

2.3 Extraction of anthocyanin

Anthocyanin was extracted in two different ways, using sterilized distilled water and acidified methanol extraction. Some fresh red cabbage leaves were chopped into tiny pieces which were placed in a beaker and covered with distilled water, about one and half times their volume, the sample was heated to 50^0C and maintain for 10-15 minutes.

1% HCl-Methanol was prepared (99 parts methanol one part concentrated HCl) and chopped red cabbage was ground with a pestle with minimal volume. The extraction was allowed to occur over night in a dark refrigerator. The colored liquid was filtered and was extracted with chloroform at 4000 rpm at 4^0c. The extract was stored in amber colored bottle.

2.4 pH experiments.

The cabbage extract was mixed with equal volume of various solutions of different pH(1.0-14.0) The extracts mixed with H_2SO_4 and NaOH, were titrated against each other, which lead to change of color of anthocyanin at the titration point, which proves that this is not an artifact.

2.5 Detecting monomeric anthocyanin pigment concentration.

The concentration of monomeric anthocyanin was detected using pH differential method (Francis, Fluleki, et al., 1968) Anthocyanin was extracted in two different pH solutions, 1.0 and 4.5. 1% HCl-methanol was used as the blank solution and the readings were taken, using shimadzu_UV mini 1240, at 520nm and 700nm.The readings were substituted in the equation for monomeric anthocyanin pigment concentration;

$$\text{Concentration} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L}$$

where A is the absorbance {A = (A_{510}-A_{700}) pH 1.0 –(A_{510}-A_{700})pH 4.5}, which gave a value of 0.2, MW is the molecular weight; 449.2 gm/mol^{-1}, DF is the dilution factor which is 1, ε is the

literature extinction co-efficient which is 26900 L $cm^{-1}mol^{-1}$ and L is the path length which is 1 here.

2.6 Checking the purity of extract using paper chromatography.

We used high quality chromatography paper . Two types of mobile phases were used, mobile phases: 1-butanol: acetic acid: water (4:1:5) and acetic acid: HCl: water (5:1:5)

Leaving 1cm from bottom and top portion, (which are respectively sample front and solvent front) the crude sample was spotted with a capillary on the marked sample front, allowed it to air dry and was introduced into the chromatography jar with the help of a twine, in such a way that only the ends of the paper was in contact with the mobile phase. The jar was closed and was made sure it was air tight The set-up was left until solvent had rised up to the solvent front (approximately 3 hours). The solvent rises the stationary phase, by capillary action because of the fibrous nature of cellulose. The resultant paper was air dried and was further used to visualize bands and calculate Rf value.

2.7 Reducing assay

This assay needed anthocyanin extracts at different dilution in1% HCl-methanol solution, Phosphate buffer of 200mM (pH 6.6), 1% potassium ferricyanide, 10% trichloroacetic acid 0.1% ferric chloride, test tubes, water bath at 50^0C, pipettes and tips, UV-VIS spectrometer, quartz cuvettes and distilled water. Cabbage extract in 1ml of methanol were mixed in separate tubes with 2.5ml of phosphate buffer and 2.5ml of 15 potassium ferricyanide .The tubes were placed in boiling water bath for 20 minutes at 50^0c.It was then cooled rapidly and mixed with 2.5ml of 10% trichloroacetic acid and 0.5ml of 0.1% Ferric chloride. The amount of iron-ferricyanide complex formed was determined by measuring the formation of perl's Prussian blue at 700nm after 10 minutes .The increase in absorbance of the reaction mixtures increased reducing power.

2.8 Antimicrobial activity

The antimicrobial activity was estimated by paper disc diffusion method using cabbage extract in acidified methanol, at various concentrations, in bacterial strains plated on LB agar (*E.coli, Bacillus, Staphylococcus, Streptococcus, Pseudomonas, Salmonella)* and fungal strains plated on SDA agar plates (*Aspergillus niger, Fusarium, Alternaria, Rhizopus).*.The discs were aseptically dipped in the extract for 2 hours, dried and were carefully and firmly placed simultaneously on the LB agar plates ((Luria-Bertani medium) : 1.0% bactotryptone, 1.0% NaCl, 0.5% bacto-yeast extract ; pH adjusted to 7.0 with 0.1N NaOH, 1.5% (w/v) bacto agar) seeded with bacterial suspension; SDA plates with fungal cultures. Filter paper discs dipped in 1% HCl-methanol was used as control. Antimicrobial activity was determined by measurement of zone of inhibition around each paper discs.

2.9) Cabbage DNA isolation and gel retardation assay.

Samples were taken out from freezer and ground using mortar and pestle, by adding minimal volume of lysis buffer (CTAB, Tris-HCl NaCl, EDTA, β-mercapthoethanol). The suspension of lysed cells was placed in a water bath for 1 hour at 65ºC. The solution was cooled to room temperature and an equal volume of chloroform was added and gently mixed by slowly turning

over the tube for 10 minutes. The two phases were separated by centrifugation at 13,000rpm for 15minutes at room temperature. The viscous aqueous phase was transferred to a clean centrifuge tube. The pooled aqueous phases were precipitated with Isopropanol. The precipitated DNA was dissolved in TE (10mM Tris-HCl (pH 8.0), 1mM EDTA) buffer after a number of times of alcohol wash. The absorbance of the DNA was measured at 260nm and 280nm. The ratio of A_{260} to A_{280} was found to be greater than 1.75.

The isolated high molecular weight genomic DNA was loaded in 0.8% agarose gel in TAE buffer(2M Tris base, 0.5M EDTA, pH adjusted to 8.0 with glacial acetic acid), mixed with an appropriate volume of 6X gel loading dye (30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) and 0.5µg Ethidium bromide/ml of TAE. It was run at 50-100 volts/cm.

For Gel retardation assay/EMSA, similar gels were casted with wells at centre. The DNA samples were incubated on ice, with appropriate concentrations of anthocyanin extracts for 30 minutes. The gel was run at 50 volt/cm, at 16^0C, for 4 hours. The gel was viewed on UV trans illuminator without EtBr staining, for recognizing the orientation of the movement of anthocyanin pigment. Later stained gel was viewed under UV to detect whether the pigment has interaction with DNA.

3 Results and Discussion

3.1 Test for pH activity.

An indicator can be defined as a substance which indicates the presence or absence of another substance or the degree of a certain reaction through characteristic changes, especially colour and anthocyanin can be used as a ph indicator. Anthocyanin was extracted using water and 1% acidified methanol, and this was added to test tubes containing solutions of different pH.

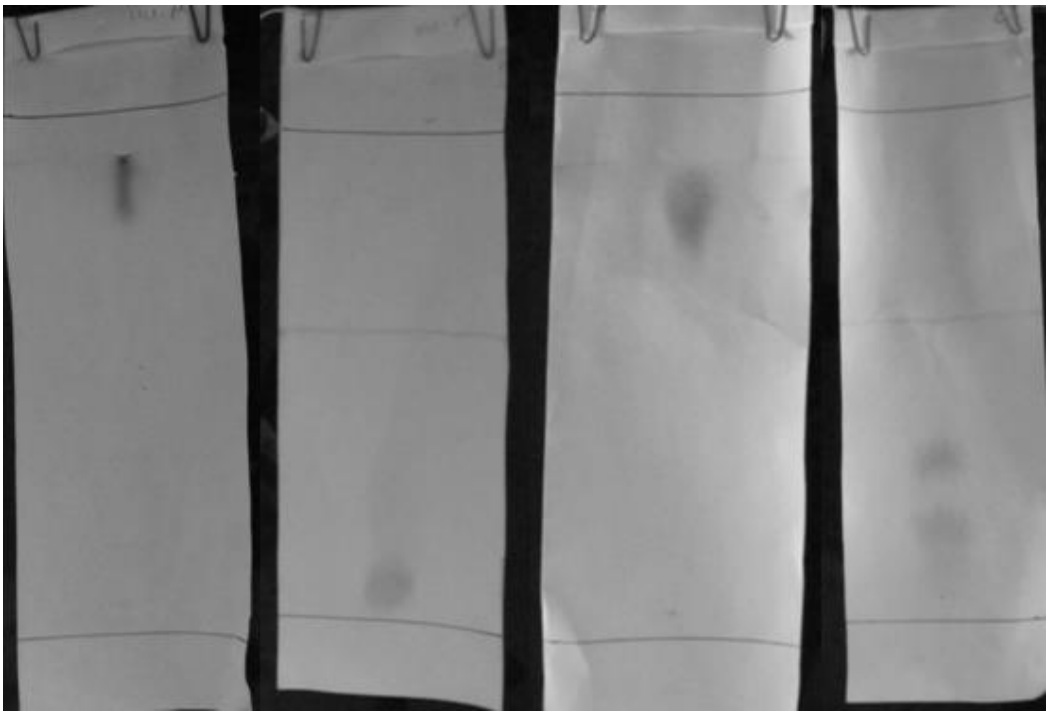
To confirm that, this was not an artifact, a titration test was also conducted. 1 N Sodium Hydroxide, (NaOH), was titrated against 1M Sulphuric acid, (H_2SO_4). Titration point was found to be 31.3ml, where the red colour indicating acidic pH turned to purple colour representing neutralization.

*3.2 Paper chromatography to check the purity of extracted anthocyanin***.**

As we had obtained anthocyanin extract, in water as well as acidified methanol in order to check, which one was pure, we did paper chromatography. Here 2 types of mobile phases were used, acetic acid: HCl: water (4:1:5) as Mobile phase I and 1-butanol: acetic acid: water (5:1:5) as Mobile phase II. Non-polar molecules move faster than the polar ones. The migration is governed by by their relative solubilities in the polar stationary phase and non-polar mobile phase, which can be estimated by calculating, a dimensionless term, R_f value (Table 1).

$$R_f = \frac{\text{Distance traveled by the sample}}{\text{Distance traveled by solvent front}}$$

Here as anthocyanin itself has colour, there was no need of other colouring reagents. The purity of bands could also be checked under UV (Figure 2).



A B C D

Figure 2 Paper chromatography of anthocyanin extracts, run using different mobile phases

Samples	R_f value
A	0.9236
B	0.2696
C	0.838
D	0.208

A- HCl methanol extract in mobile phase I
B- HCl methanol extract in mobile phase II
C- Crude extract in mobile phase I
D- Crude extract in mobile phase II

Table1: R_f values of anthocyanin extract

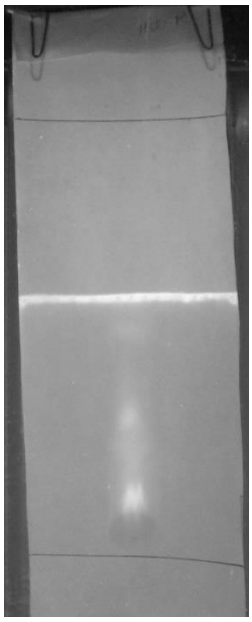


Figure 3: one of the chromatogram, under UV

From the above results we can infer that, migration rate of mobile phase I (acetic acid: HCl: water), was fast, but this effected the resolution while purification. At the same time mobile phase II (1-butanol: acetic acid: water) is having a slow rate, and helps in purification.

The extract will be containing many anthocyanins, loosely conjugated sugars and proteins, betain etc, which cannot be found using naked eye. But we can identify anthocyanin, if we observe it under UV, as it can absorb UV radiations (Figure 3). It is also clear that the HCl-methanol extract is much more pure than that of crude extract.

3.3 Concentration check of extracted anthocyanin

From the literature, it was sure that the pigment has got two λ-max at 260nm and 500nm, but we did an absorption spectrum at various wave lengths (nm), using shimadzu_UV mini 1240, to make sure (Figure 4). The result has been plotted graphically below (figure: 3.1); the blank used was 1% HCl-methanol solution.

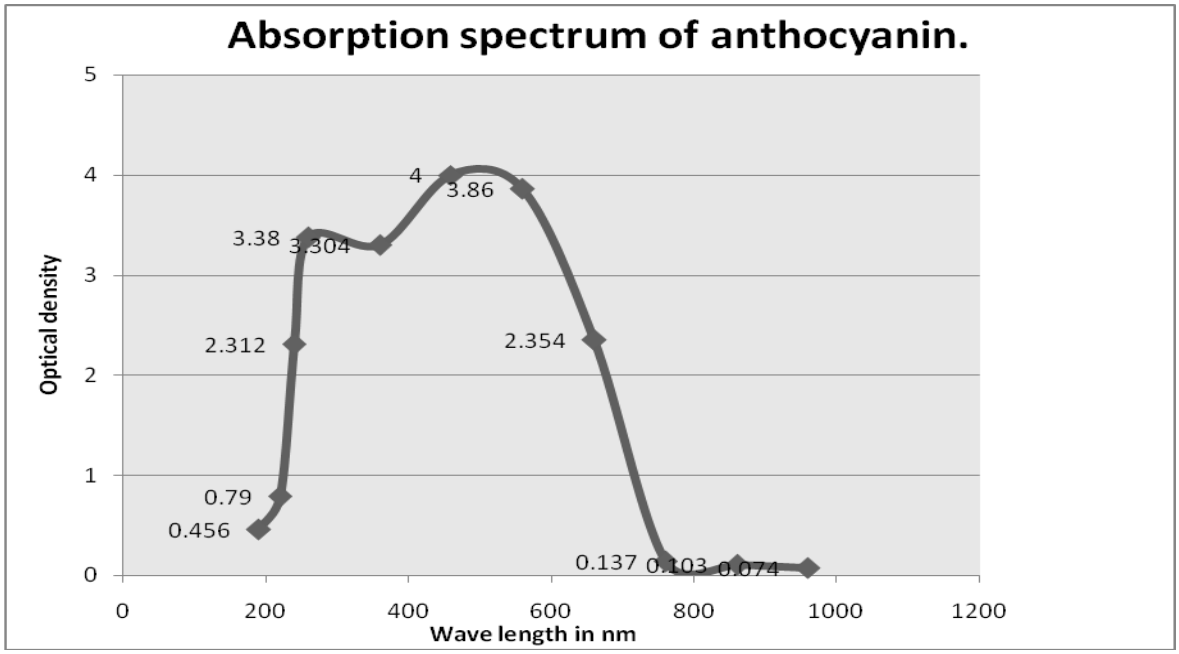


Figure 4: Absorption spectrum of anthocyanin

Using pH differential method (Francis, Fluleki, et al., 1968), concentration was checked, using the equation for monomeric anthocyanin pigment concentration;

$$\text{Concentration} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L}$$

where A is the absorbance {A = (A_{510}-A_{700}) pH 1.0 –(A_{510}-A_{700})pH 4.5}, which gave a value of 0.2, MW is the molecular weight; 449.2 gm/mol^{-1}, DF is the dilution factor which is 1, ε is the literature extinction co-efficient which is 26900 L $cm^{-1}mol^{-1}$ and L is the path length which is 1 here. The principle of pH differential method is that, monomeric anthocyanin pigments change colour reversibly with pH, i.e, at pH 1.0 oxonium ion exists in a coloured state, and at pH 4.5, it becomes a colourless hemiketal. Absorption at 510 nm is proportional to the concentration and at 700 nm measures the grid value/turbulence. Polymeric degraded anthocyanin is not included in this method as they are resistant to colour change.
The concentration of the extraction was found to be, 3.34mg/ml of anthocyanin.

3.4 Antimicrobial Activity

Antibacterial activity was checked on LB agar, with obtained 6 strains of bacteria; *B.subtilis, E.coli, Staphylococcus, Streptococcus, Pseudomonas* and *Salmonella*, using discs soaked in anthocyanin (Figure 5). Antifungal activity was checked on SDA agar, with obtained 4 strains of fungi, *Alternaria, A. niger, Rhizzopus* and *Fusarium*, using the same way as mentioned above.After an overnight incubation, zone of inhibition was taken in millimeter, to estimate sensitivity (Figure 6).

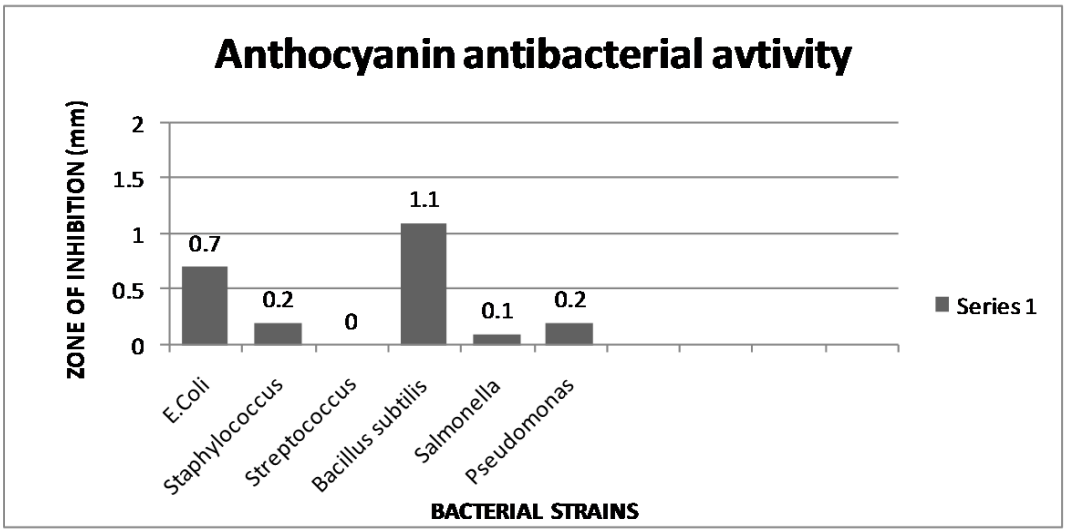


Figure 5: A histogram showing sensitivity of bacteria against anthocyanin

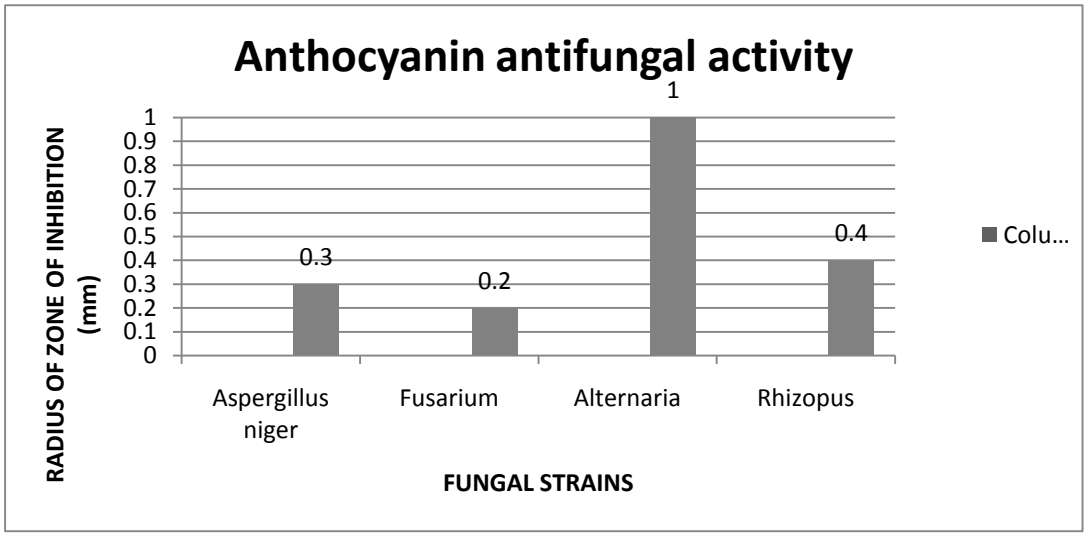


Figure 6: A histogram showing sensitivity of fungi against anthocyanin

Bacillus subtilis and *Alternaria*, showed to have the highest sensitivity to anthocyanin extract.

3.5 Ferric Reducing Assay

In order to find the reducing potential of anthocyanin, the following test was conducted. It was noted that as the anthocyanin concentration increased, absorbance was also found to increase. This was due to the formation of iron (II) - ferricyanide complex and was determined by measuring Perl's Prussian blue at 700nm (Figure 7).

The graph shown below indicates the reducing power of anthocyanin, and this reflects the anti-oxidant property and radical scavenging activity. Thus anthocyanin plays an important role in the routine diet.

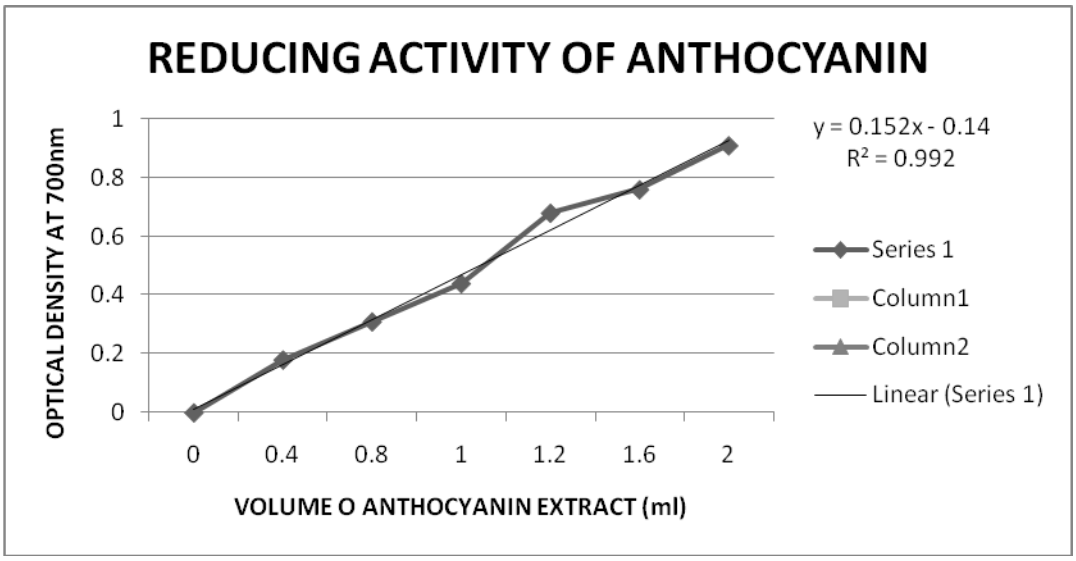


Figure 7: Graph showing reducing power of anthocyanin

3.6 Isolation of DNA from Red Cabbage and EMSA

DNA was isolated from red cabbage, checked its purity on gel and UV spectrometer, taking the 260:280 ratio, which was 1.78 for obtained DNA. EMSA (Electron Mobility Shift Assay) or GR (Gel Retardation) assay is usually done to find interactions between DNA and proteins/ proteins and proteins. But we know that anthocyanin has antifungal property and as its regulation/biosignalling pathway has not been traced out, we thought of finding, whether anthocyanin has a property of binding DNA, in presence/absence of fungal elicitors and can it alter the expression in red cabbage. There are chances that anthocyanin may act as activators of promoters that control anthocyanin synthesis. It has also been reported that during production of anthocyanin *invitro*, it has been found that, fungal infection increases anthocyanin production in the callus. The following figure has been done at pH 7.0.

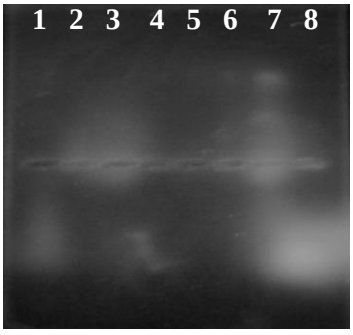


Figure 8: EMSA gel, showing affinity of anthocyanin with DNA

No. of lanes	Samples loaded/composition
1	20µl DNA+ 5µl gel loading dye
2	25µl of anthocyanin crude
3	25µl of anthocyanin in HCl-methanol
4	-
5	-
6	-
7	20µl of anthocyanin + 5µl of DNA
8	-

Table 2: Lanes in agarose gel

From the 7th lane, we can find that anthocyanin is moving towards positive electrode, carrying a scarce amount of DNA. So, it means anthocyanin has got some properties of binding DNA. Anthocyanin gives a blue fluorescence and DNA gives the orange fluorescence, so it was easy to detect by visualization under UV transilluminator.

When the same experiment was repeated at various pH, and the best affinity was obtained at pH 7, because the charge of anthocyanin changes with pH. Another point to notice is that, anthocyanin exists at pH-3.5 inside the vacuoles but at acidic pH the agarose may not polymerize.

4. Conclusions

From literature it is evident that anthocyanin genes have been evolved lately. It is not found in any lower plants or micro organisms. So it is expected that due to the UV penetration the anthocyanin genes must have evolved because they act as a sunscreen, a genoprotectant, pollinator attractant, repellent for grazers and also an active pigment which has got a wide range of absorption spectrum. It has also been seen that, anthocyanin expression has increased during fungal infections and it shows its pivotal role in plant defense. It can also be used in Dye Sensitized Solar Cells (DSSC). Scientists have been trying to synthesize anthocyanin pigments using prokaryotic systems and many patents have been filed to synthesize anthocyanin artificially. This shows the importance of anthocyanin in human diet as well as in other fields. So in this paper we have tried to characterize anthocyanin from red cabbage, which is less conjugated with sugar moieties. As well as to find out the affinity of the extracted pigment to DNA, while fungal infections.

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Response of *Chromolaena Odorata* (L.) King & Robins to Urban Air Pollution: Effects on Anatomy, Leaf Extract pH, Chlorophyll and Relative Water Content

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Abstract

The present study investigates the impact of urban air pollution from automobile exhaust on a common roadside plant *Chromolaena odorata* (L.) King &Robins (*Eupatorium odoratum*). Parameters studied were anatomy of stem and leaves, stomatal index, stomatal pore size, total chlorophyll content, leaf extract pH and relative water content. Decrease in the diameter of xylem vessels in the stem and leaves, stomatal index, stomatal pore size and total chlorophyll content were noticed in the plant grown in the polluted area. Leaf extract pH and relative water content were more in the polluted plant as compared to the control plant taken from pollution free area. The results obtained reveal that *Chromolaena odorata* is less susceptible to the harmful effect of automobile exhaust pollution.

Key words: Chromolaena odorara, urban air pollution, stomatal index, stomatal pore size, chlorophyll content, leaf extract pH, relative water content

1. Introduction

Air pollution has become an extremely serious problem for the modern industrialized world. Vehicle exhaust emissions are a dominant feature of urban environments and are widely believed to have detrimental effects on plants. It has been observed that the survival of plants in the polluted atmosphere is correlated with structural and metabolic adaptations to the stressful environmental conditions. Much experimental work has been conducted on the analysis of air pollutant effects on crops and vegetation at various levels ranging from biochemical to ecosystem levels. The use of plants as monitors of air pollution has long been established as plants are the initial acceptors of air pollution.

Before the appearance of external visible symptoms, there appears many physiological changes in plants as a result of the exposure to various pollutants (Dohmen, *et al.,* 1990). Impact of air pollution on the chlorophyll content of plants has been analyzed by Agbaire & Esiefarienrhe (2009) and Joshi & Swami (2009). Histo-anatomical studies and investigations regarding the modifications that occurred in trees' leaves under the effect of air pollution have been studied by Da Silva *et al*. (2005), Maranho *et al*. (2009) and Gostin & Ivanescu (2007). Studies have also conducted on the impact of air pollution on the leaf extract pH (klumpp *et al*., 2000), ascorbic acid content (Hoque *et al*., 2007) and relative water content (Rao, 1979). All these parameters are helpful in the estimation of tolerance levels of plant species to various pollutants (Liu & Ding, 2008). Studies have also been undertaken on the variations in the stomatal index and stomatal pore size due to air pollution (Garg & Varshney, 1980; Weyers & Travis, 1981; Singh *et al.,* 1995).

The present investigation analyzes the response of *Chromolaena odorata* (*Eupatorium odoratum*), a common road side Asteracean member with worldwide distribution, to urban air pollution.

2. Materials and Methods

2.1 Area of study

Area of study is the Thrissur Town in Kerala, India where the air is polluted mainly from the emissions from automobiles**.** This study area was designated as the experimental site (ES). Three replicates of fully matured plants were collected randomly from the road sides and immediately taken to the laboratory for analysis. A site at rural area of Thrissur district with a pollution free air and with similar ecological conditions was selected as the control site (CS).

Impact of air pollution from vehicle exhaust on the experimental plant *Chromolaena odorata* was analyzed by studying the following parameters:

- anatomy of leaves
- anatomy of stem
- stomatal index
- stomatal pore size
- chlorophyll content
- Leaf extract pH and
- Relative water content

2.2 Anatomy of stem and Leaves

For the study of leaf and stem anatomy, leaves from second node and tender stem from second inter node were selected for taking free hand sections. Sections were stained with saffranine, mounted in glycerine and observed under microscope. Photographs of the sections were taken with trinocular microscope attached with Nikon D60 digital camera. Diameter of xylem vessel was measured with a micrometer.

2.3 Stomatal index (SI)

Stomatal index (SI) of both upper and lower epidermis was calculated using the formula:

$$S.I = \frac{S}{S+E} \times 100$$

Where

S.I-Stomatal index

S -Number of stomata/unit area

E -Number of epidermal cells/unit area

2.4 Stomatal Pore Size

Length and width of stomatal pore was measured using micrometer.

2.5 Total Chlorophyll content

Arnon's method (1949) was adopted for estimating total chlorophyll content. Absorbance of chlorophyll extract in 90% acetone was measured at 645 nm, 652nm, and 663nm using a spectrophotometer.

2.6 Leaf Extract pH

Leaf extract for the determination of the pH was obtained by homogenizing 5 gm of fresh leaves in 10ml deionized water and pH was determined by using pH meter.

2.7 Relative Water Content

Relative water content of the leaves was estimated following the method described by Singh (19 77) and calculated using the formula:

RWC = [(FW –DW)/ (TW –DW)] x 100

where, FW = Fresh weight, DW = dry weight and TW = turgid weight.

Results obtained were compared with those obtained for the control plant taken from unpolluted area. Both the control and pollution exposed plants were in their reproductive stage during the study period.

The data obtained were analyzed statistically using one-way ANOVA

3. Results and Discussion

Observations made on the plant anatomy have shown that air pollution has caused a decrease in the size of xylem vessels by 27% in stem and 17% in the leaf (table 1, fig.1). The stomatal index recorded in the plant material collected from polluted area was 18% more than that of control in the lower epidermis while it was 50%less in the upper epidermis than that of unpolluted sample. A significant decrease in the pore width of stomata was noticed in the polluted sample compared to the control plant (table 1, fig.2)

Parameter	Site	
	Control (CS)	Experimental (ES)
Diameter of xylem vessel (µm)-stem	40.87±0.84	29.74±1.24*
Diameter of xylem vessel(µm)- leaf	30.16±1.01	24.96±0.84**
Stomatal Index- upper epidermis	23.37±0.42	11.71±1.1*
Stomatal Index- lower epidermis	22.34±1.12	27.45±1.1**
Stomatal pore length (µm)-upper epidermis	79.04±1.32	73.04±1.0**
Stomatal pore length (µm)-lower epidermis	79.04±1.35	76.96±1.89
Stomatal pore width (µm)-upper epidermis	23.92±0.5	10.4±0.8*
Stomatal pore width (µm)-lower epidermis	22.88±1.0	14.56±1.01*

Table 1. Impact of Urban air pollution on the stem and leaf anatomy of *Chromolaena odorata*

Stomatal pore width showed a decrease of 58% and 36% in the upper and lower epidermis respectively. Total chlorophyll content was decreased by 26% when the plant was exposed to vehicle exhaust pollution. The leaf extract was alkaline in nature with pH 8.98 in unpolluted sample, that increased to 9.60 in the polluted plant. Relative water content in the polluted sample registered an increase of 7.2% over that of unpolluted control plant (table 2).

Parameter	Site	
	Control (CS)	Experimental (ES)
Total chlorophyll content (µg/ml)	16.39±0.9	12.08±1.15**
pH of leaf extract	8.98±1.19	9.60±1.26
Relative water content (%)	89.88±0.07	96.89±0.45

*significant at $p<0.01$

**significant at $p<0.05$

Table 2. Changes in the total chlorophyll content, pH of leaf extract and relative water content of *Chromolaena odorata* exposed to urban air pollution

Impact of air pollution on the histo-anatomy of different plant species has been studied by many workers (Da Silva *et al*., 2005; Maranho *et al*.,2009; Gosten and Ivanescu,2007). Decrease in diameter of xylem vessel due to heavy deposition of wall material as observed in the current investigation, decreases the water conductivity capacity of the plants thereby reducing their growth potential. The modification of the frequency and size of stomata as response to the environmental stress is an important feature as it controls the absorption of pollutants by plants. According to Varma *et al*. (2006) the decrease in stomatal pore size in the polluted environment may be an avoidance mechanism of the plant against the inhibitory effect of pollutants on physiological activities such as photosynthesis. However, increase in the stomatal index in the lower epidermis in polluted species is not a common feature of plant species exposed to air pollution.

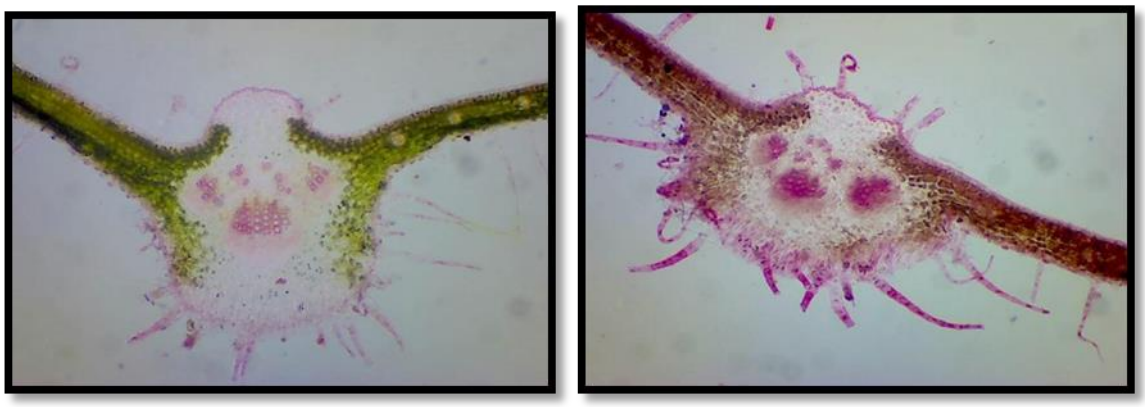
Chlorophyll content of plants signifies its photosynthetic activity as well as the growth and development of biomass. It is well evident that chlorophyll content of plants varies from species to species, age of leaf and also with the pollution level as well as with other biotic and abiotic conditions (Katiyar and Dubey, 2001). A decline in the total chlorophyll content was noticed in *Chromolaena odorata* grown in polluted area indicating the sensitive nature of the plant to the pollutants.

Leaf extract pH registered an increase of 6.4% over that of unpolluted control plant. The changes in leaf-extract pH might influence the stomata sensitivity to air pollutants. High pH may increase the efficiency of conversion of hexose sugar to ascorbic acid and it is related to the tolerance to pollution (Lui and Ding, 2008; and Escobedo *et al.*, 2008).

Relative Water Content (RWC) of a leaf is the water present in it relative to its full turgidity. Decreased RWC results from increased loss of water and dissolved nutrients from the cells, and leads to an early senescence of leaves (Agrawal and Tiwari, 1997).Relative water content was slightly higher in polluted conditions as compared to the control plant indicating the tolerant nature of the experimental plant to the pollutants. Similar result was obtained by

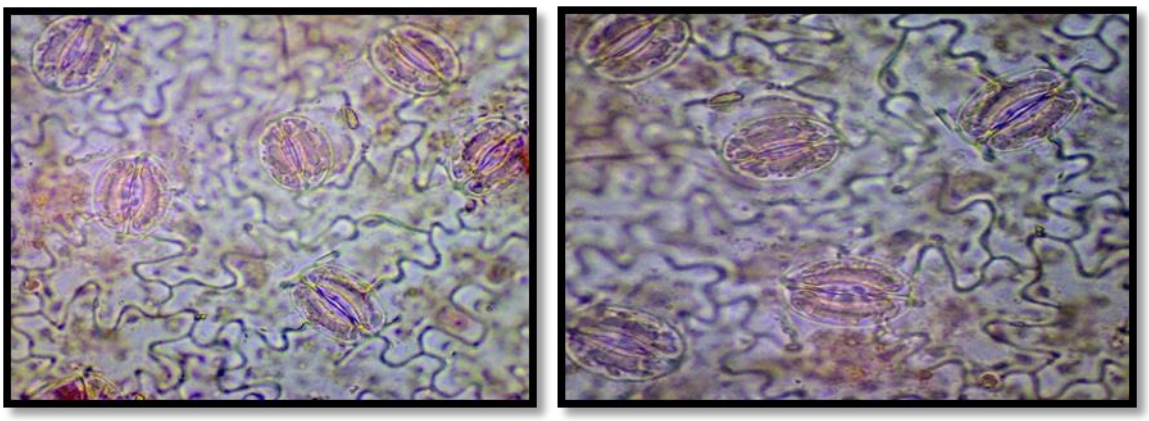
Chandawat *et al*. (2011) in various species of *Ficus* like *F. religiosa,F. benghalensis and F. glomerata.*

Most of the parameters studied in the current investigation revealed the tolerant nature of *Chromolaena odorata* to the pollution from automobile exhausts. The histological modifications which occurred in the polluted atmosphere may potentially be used as biological markers for air pollution presence. At elevated concentrations, air pollutants are toxic not only for plants but also for man. Therefore, understanding the impact of pollutants on terrestrial ecosystems is important for several reasons, and one of these being the utilization of vegetation to improve air quality by the removal of pollutants particularly in the densely populated areas.



Control Polluted

Fig.1: Impact of urban air pollution on the leaf anatomy of *Chromolaena odorata*



Control Polluted

Fig.2: Impact of urban air pollution on the stomatal characters of *Chromolaena odorata*

4. Conclusions

The results obtained for various parameters suggest that *Chromolaena odorata* (L.) King & Robins is less sensitive to air pollution from automobile exhaust.

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Induced breeding and hatchery development of *Anabas testudineus* (Bloch)

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ABSTRACT

Ovaprim were injected intraperitoneally into male and female *Anabas testudineus* at dose of 0.05ml per kg of body weight. This dose found to be effectively induced ovulation. Without stripping gametes of both sexes fertilization were successful and produced viable young ones. The egg yolk of the larvae was fully absorbed two-days after hatched and they started to eat newly hatched *Artemia.* Their mouth opening on the 2-day after hatching is measured 167.81µm and reached 300.85µm on the 13-DAH (days after hatched). The use of the climbing perch as a genetic model has moved beyond the proof-of-concept for the analysis of vertebrate embryonic development to demonstrated utility as a mainstream model organism for the understanding of human disease. The hardy nature of climbing perch is exploited by the toxicology studies and behavior assays.

Key words: *Anabas testudineus*; early rearing; spatial learning.

1. Introduction

Commonly found freshwater fish *Anabas testudineus* (Bloch**)** popularly known as 'climbing perch or climbing gouramy'. The common name climbing perch originated from the legend that *A. testudineus* climb palm trees to suck juice. Probably the origin of this myth is that birds pick this fish when it travels over land and place it on palm trees (Norman, 1975). Climbing perch, *Anabas testudineus*, Bloch (1792) inhabits mostly in canals, lakes, ponds, swamps, medium to large rivers, brooks, flooded fields and stagnant water bodies including sluggish flowing canals, especially those ponds in low-liying places, where the ponds cannot be drained completely (Bian, 1969). They are hardy and it can thrive in oxygen depleted water bodies (Pethiyagoda, 1991). They are well known for their ability to migrate between ponds over land (Sterba, 1983; Liem, 1987; Davenport and Abdul Matin, 1990; Sakurai *et al*, 1993). Usually climbing perch adopts a near- horizontal posture and alternately drives the left and right spiny sub operculars into the substratum, using the tail to vault over the sub opercular (which acts in the manner of a short vaulting pole) It possesses a special accessory air breathing organ, situated just above the gills in a large extension on the upper part of each gill chamber, which facilitates the utilization of atmospheric air for their respiration (Graham, 1997). This species also been reported as one of the successful biological control organisms in controlling mosquitoes like *Aedes sp.,Culex sp. and Anopheles sp.* in sewage waters (Chandra *et.al.*, 2008) According to Shinsuke *et al.*, (2008) this species is widely distributed, including India, the Indochinese Peninsular, southern China, Taiwan, the Philippines and Indonesia. It feeds on macrophytic aquatic vegetation, shrimps and fish fry (Pethiyagoda, 1991). This fish

can tolerate extreme unfavorable water conditions and often seen associated with turbid and stagnant waters.

Climbing perch is a potamodromous fish (Riede, 2004) that undertake lateral migration from the permanent water bodies to flooded areas during the rainy season and return to permanent water bodies at the end of flood (Sokheng *et al.* 1999). At times, it may end up on the land and crawl on the moist soil to reach back to the pond. This fascinating behaviour shows the acute spatial cognition of the fish. It possesses accessory respiratory organ with which it can breathe in hypoxic harsh conditions. The accessory respiratory system includes labyrinthine organ and the respiratory membrane covering the supra brachial chamber. This fish is able to survive for several days or weeks out of water if the air breathing organs are kept moist. During the dry season it lives buried in the mud and passes into a resting stage of aestivation similar to that observed in the African lung fish (Thirphan, 1984). Reported to undertake lateral migration from the mainstream, or other permanent water bodies, to flooded areas during the flood season and return to the permanent water bodies at the onset of the dry season. This obligatory air-gulping fish can be found in most drainage systems and is considered to be a hardy species since it can tolerate extremely hostile conditions (Atack, 2006). At present, it is one of the primary freshwater/ brackish water food and ornamental fish in this region. It is usually marketed alive fetching a price of around 40 Rs/kg. They are also well known for their taste, high nutritive value, and recuperative and other medicinal qualities. As a popular fresh water species, the climbing perch has been used in developmental biology for many years.

The knowledge from the field of fish cognition has wider application in conservation of endangered species. The fish larvae born and brought up in homogeneous hatchery condition fail to develop cognitive abilities like predator recognition and development of anti predator responses (Brown and Day, 2002). Due to their inability to learn the novel stimulus in the new ecosystem these fish will not be able to cope up with the harsh and novel conditions present in the natural ecosystems where they are released as a part of restocking and reintroduction programmes (Brown and Laland, 2001).So a study on induced breeding is indeed as a preliminary step to generate more information on their biology, breeding patterns and to promote the post induced breeding operation to promote viable young one to nature.

2. Materials and methods

Climbing perch mature at approximately 70-100 mm in size and breeds in paddy fields and seasonal ponds with water depth of at least 10-25 cm depth. Unlike other anabantid species, they do not build bubble nests or care for their eggs, which float water surface (Axelrod *et al,* 1971; Sakurai *et al,* 1993). During the larval and juvenile stages they prefer plankton and in adult stages they are omnivorous and mainly feed on insects, other invertebrates, fishes and plants (Singh and Samuel, 1981; Riehl and Baensch, 1991). Females can be induced to spawn and a single clutch may contain several hundred eggs. Generation time is short, typically 4-6 months. Climbing perch eggs are large (0.6 mm in diameter at fertilization) and optically transparent, the yolk being sequestered into a separate compartment. As fertilization is external, live embryos are accessible to manipulation and can be monitored through all developmental stages under a dissecting microscope. Development is rapid, with precursors to all major organs developing within 36 h and larvae exhibit foraging behaviours within five days post fertilization, i.e. 2-3 days after hatching.

2.1 Rearing conditions and induced breeding

Healthy adult breeders were collected from pond near Avittatur (10°25' 10° 18 47"*N* latitude 76°17’19 76°12'48"E longitude) located in Kerala during April-March 2009, and housed in large cement tanks for acclimatizing with the laboratory conditions. Fish feed pellets (Marvel aquarium fish feeds India Ltd.) were provided *ad libitum* for a two-week settling period and water was changed twice in a week. In the June 2009, at monsoon one female (10cm) with bulged belly and two matured milt oozing males (8cm) were selected from the brood stocks for induce breeding. The breeders were given intramuscular injections of Ovaprim hormone (0.5 ml/kg fish) near the caudal peduncle. Then, all the brood stock were released into three breeding containers which the ratio of female to male was 1:2 until fertilization occurred. After 7-8 hours, the breeder released their fertilized eggs and the eggs hatched after 25-27 hours. The temperature during incubation period was set at 26-29C°. Within 12 hr of hatching of the eggs, breeders were removed from their breeding tank and larvae were reared in group under different rearing environments according to the experiment setup.

Eggs that fertilized were observed each hour after fertilization to determine all the stage of embryonic development under the microscope and photographs was taken. For the first 2 hours, it was observed for 4 times (at half an hour interval) and after the first two hours, it was observed for at one hour interval, until the larvae hatched. After hatching the larvae were immediately transferred into the 500ml beakers with 20 larvae in each beaker. The larvae were fed on freshly hatched nauplii *Artemia salina* during the 1-7 days and combination with powdered fish feed pellets during day 8-30 days. A total of 30 larvae were sample early in the morning before feeding and measured the length and weight of the larvae until they become juveniles.

Fig A

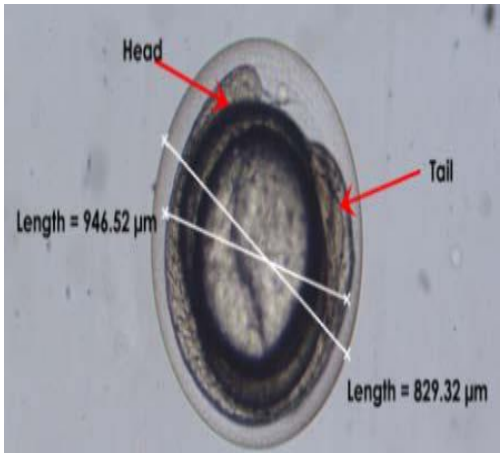

Fig B

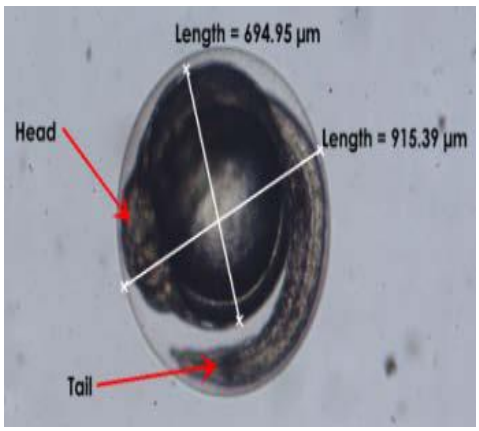

Fig C

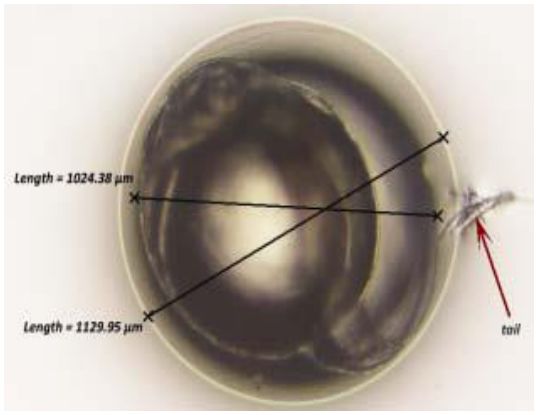

Fig D

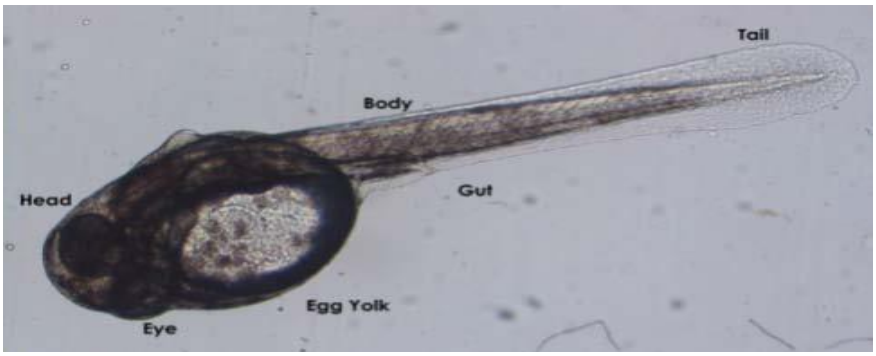

Fig E.a

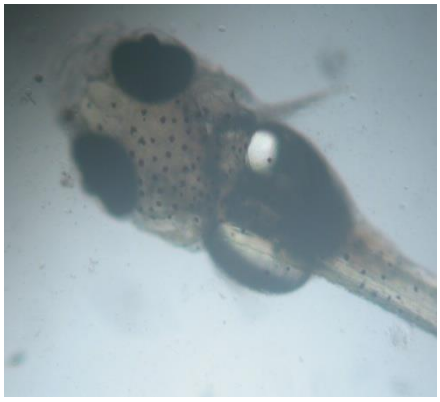

Fig E.b

Fig E.c

Fig. 1 (A) formation of yolk plug which resembles a belt surrounding the yolk sack (B) the head and tail were fully formed (C) the tail of larvae were split from the yolk sack and the movement of tail and heart were clearly seen (D) the tail was the first organ that came out from the eggs at the time of hatched. (E) a, b, c. Newly hatched of *A.testudineus.*

3. RESULT

The fertilized eggs of climbing perch were white-cream in colour, bright and clear in appearance, round in shape and epipelagic (floating on the surface of water).The female released about 50000- 60000 eggs which are fertilized by male. The embryonic developments of *A.testudineus* are shown in figure 1. Larvae moved actively after 23-25h after fertilization.

The movement of the tail struck against the head end at the egg shell causing the egg to break. The tail is the first organ that came out from the eggs and followed by the other organs. All the larvae in aquarium were fully hatched 28h. The temperature at the time of hatching is 25-27°C. The hatching of the larvae is inversely related to temperature. After hatching, the larvae have non-pigmented eyes, undeveloped mouth and their fins were not fully developed. Only the gut and anus was well formed. The larvae were seen like school and attached on the surface of aquarium's walls. The average total length of newly hatched larvae was 2.94mm and the mean total length at 1st day after hatched was 3.98mm. After the 12h after hatching, the mouth was formed. The eye of *A.testudineus* larvae developed 24h after hatching. The egg yolk of the larvae was fully absorbed two-days after hatched and they started to eat newly hatched *Artemia*. Their mouth opening on the 2-day after hatching is measured 167.81µm and reached 300.85µm on the 13-DAH (days after hatched). They are given combination food of *Artemia* with powdered dry food on the 14-DAH and fully given powdered dry food on the 21-DAH.

The yolk-sac larvae development stages of *A.testudineus* oocurs from hatching until the egg-yolk have fully absorbed on day 5 DAH. From the 5-7 DAH *A.testudineus* larvae have reached the development stage of preflextion larvae. Then, the stage was followed by flexion larvae development and *A.testudineus* larvae have reached this stage on the 10-14 DAH. On the 18-21 DAH, the *A.testudineus* larvae were at the post flexion larvae stage were at this stage the formation of the caudal fin (hypural elements vertical) to attainment of fully external meristic complements (fin rays) could be observed. The juvenile stage finally appears when the completion of the fin ray counts and beginning of squamation occur until fish enter adult's population or attain sexual maturity. The *A.testudineus* larvae reached juveniles stage on the 25-28DAH.

The use of the climbing perch as a genetic model has moved beyond the proof-of-concept for the analysis of vertebrate embryonic development to demonstrated utility as a mainstream model organism for the understanding of human disease. These studies highlighted the potential use of the climbing perch for large-scale chemical genetic screens for small molecules that perturb specific aspects of organogenesis, pattern formation and neural genesis.

4. Conclusions

In the context of behavioural studies, modified rearing environments promote adaptive behaviour that might otherwise not develop in typical hatchery environments. In this context development of a life skill training protocol for the hatchery reared fish based on the cognitive abilities of the fish is essential to control species loss from natural water bodies. It was found that *Anabas testudineus* when exposed to spatial heterogeneity during early rearing were bolder; they were also faster at seeking food than fish (Sheenaja and Thomas, 2011). From hatchery environment when they are released to nature it will be beneficial to them. It implies that environmental enrichment can improve learning abilities, as complexities in the environment provide greater sensory feedback to the brain than unstructured enclosures, resulting in increased neurogenesis. Hence, spatial learning protocols could realistically be applied on a large scale to enhance the viability of hatchery fish prior to their release into the wild.

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Nanofluids – Next Generation Coolants With Applications

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Abstract

Nanofluids, in which nano-sized particles (typically less than 100 nanometers) are suspended in liquids, have emerged as a potential candidate for the design of heat transfer fluids. The different processes and materials for the synthesis of nanofluids are discussed in brief. The diverse application side of nanofluids, giving special emphasis to the cooling applications is mentioned in this paper. This paper is an eye opener to the recent studies happening in the interesting and venturing world of nanofluids which are likely to be the smart coolants of the next generation and clarifies the use of nanofluids in industries and medicine instead of ordinary coolants.

Key words: Nano fluids, nano materials, coolants, applications in industry and medicine.

1. Introduction

Nanofluid is a suspension of solid nanoparticles (1-100nm diameter) in conventional liquids like water, oil or ethylene glycol. The emergence of nanofluids as a new field of nano scale heat transfer in liquids is related directly to miniaturization trends and nanotechnology. Nanofluids owe its history to the Advanced Fluids Program (AFP) at Argonne National Laboratory (ANL) that encompassed a wide range (meters to nanometers) of size regimes and eventually the wide research road became narrow, starting with large scale and descending through micro scale to nano scale, culminating in the invention of nanofluids. The goal of nanofluids is to achieve the highest possible thermal properties at the smallest possible concentrations by uniform dispersion and stable suspension of nanoparticles in host fluids. When used as coolants, nanofluids can provide dramatic improvements in the thermal properties of host fluids. The novel nanofluids enable a more efficient, effective and uniform heat removal capability for systems requiring highly accurate temperature control at high heat fluxes.

In the development of energy-efficient heat transfer fluids, the thermal conductivity of the heat transfer fluids has a specific role to play. Though numerous development efforts and considerable previous research have took place, major improvements in cooling capabilities have been constrained because traditional heat transfer fluids used in today's thermal management systems, such as, water, oils, and ethylene glycol, have inherently poor thermal conductivities, orders-of-magnitude smaller than those of most solids. It is well known that at room temperature, metals at room temperature in solid form have higher thermal conductivities than those of their fluids (Touloukian et al., 1970). The thermal conductivity of metallic liquids is much higher than those of non metallic liquids. As a consequence, the thermal conductivities of fluids that contain suspended solid metallic particles could be expected to be significantly higher than those of conventional heat transfer fluids.

2. Materials and Methods

Nanofluid is defined as a colloidal solvent containing dispersed nanometer-sized particles (~1-100 nm). Researchers have found out many materials that can be used as base fluids and nano particles. Stable and highly conductive nanofluids are produced by one step and two step production methods. Both approaches for creating nano particle suspensions suffer from agglomeration of nano particles, which is a key issue in all the technology involving nano powder. Therefore, synthesis and suspension of nearly non agglomerated or mono dispersed nanoparticles in liquids is the key to significant enhancement in thermal properties of nanofluids. Nano structured or nano phase materials made of nanometer sized particles enhanced physical properties not exhibited by conventional bulk solids. All physical mechanisms have a critical length scale below which the physical properties of materials are changed. Thus the particles smaller than 100 nm exhibit properties different from those of conventional solids. The noble properties of nanophase materials come from the relatively high surface area or volume ratio which is due to high proportion of constituent atoms residing at the grain boundaries. The thermal, mechanical, optical, magnetic and electric properties of nanophase materials are superior to those of conventional materials with coarse grain structures.

Nanoparticles used in nanofluids have been made of various materials such as oxide ceramics (Al_2O_3, CuO), nitride ceramics (AlN, SiN), carbide ceramics (SiC, TiC), metals (Cu, Ag, Au), semiconductors (TiO_2, SiC), carbon nanotubes, and composite materials such as alloyed nanoparticles $Al_{70}Cu_{30}$ or nanoparticle core-polymer shell composites. In addition to the nonmetallic, metallic and other materials for nanoparticles, completely new materials and structures such as materials 'doped' with molecules in their solid liquid interface structure may also have desirable characteristics.

Many types of liquids like water, ethylene glycol and oil have been used as host liquids in nanofluids.

Fabrication of nanoparticles can be classified into two broad categories:

1. Physical processes
2. Chemical processes

Currently a number of methods exist for the manufacture of nanoparticles. Physical method includes inert-gas condensation (IGC) and mechanical grinding. Chemical method includes chemical vapour deposition (CVD), chemical precipitation, micro emulsions, thermal spray and spray pyrolysis.

The current processes for making metal nanoparticles include IGC, mechanical grinding, chemical precipitation, thermal spray and spray pyrolysis. Sonochemical method makes suspensions of iron nanoparticles stabilized by Oleic acid. Most recently, alloyed nanoparticles $Al_{70}Cu_{30}$ was produced ball milling (Chopkar et al., 2006). In ball milling, balls impart a lot of energy to a slurry of powder, and in most cases some chemicals are used to cause physical and

chemical changes. These nanosized materials are most commonly produced in the form of powders. In powder form, nanoparticles are dispersed in aqueous or organic host liquids for specific applications.

Although nanoparticles often refer to spherical shapes, there are also various anisotropic shapes. These refer to all shapes other than spherical. Such shapes require more than one parameter to describe their shapes. The most common ones are nano rods and nano triangles. Several other shapes, such as tripods, tetra pods, stars, flowers and sheets are known, and in several cases synthetic flexibility does not exist. However, it is not possible to get most of these in the solution phase and so out of the discussion range.

Stable suspensions of nanoparticles in conventional heat transfer fluids are produced by two methods: single-step technique and two-step technique. The single-step method simultaneously makes and disperses nanoparticles into base fluids. In two-step method, we first make nanoparticles using one of the above-described nanoparticle processing techniques and then disperse them into base fluids. Most nanofluids containing oxide nanoparticles and carbon nanotubes reported in the open literature are produced by two-step process. Although the two-step method works well for oxide nanoparticles, it is not as effective for metal nanoparticles such as copper. For nanofluids containing high-conductivity metals, it is clear that the single-step technique is preferable to the two-step method.

3 MILESTONES IN EXPERIMENTAL DISCOVERIES ON NANOFLUIDS

Experimental work in nanofluids research groups world-wide has discovered that nanofluids exhibit thermal properties superior to those of base fluids or conventional solid-liquid suspensions. Studies have shown that copper and carbon nano tube (CNT) nanofluids possess extremely high thermal conductivities compared to those of their base liquids without dispersed nanoparticles and CNT nanofluids have a nonlinear relationship between thermal conductivity and concentration at low volume fractions of CNTs. The distinctive features like strong temperature-dependent thermal conductivity (Das et al., 2003b) and strong size-dependent thermal conductivity were contributed by thermal conductivity measurement experiments of nanofluids.

The potential impact of the discoveries on heat transfer applications made nanofluids promising coolants for the industrial and electronic world. As a consequence of these discoveries, research and development on nanofluids has drawn caring attention from industry and academia over the past several years.

4 Applications of Nanofluids

Nanofluids find most of their applications in thermal management of industrial and consumer products as efficient cooling is vital for realizing the functions and long-term reliability of the same. There are a large number of tribological and medical applications for nanofluids. Recent studies have demonstrated the ability of nanofluids to improve the performance of real-world

devices and systems such as automatic transmissions. This paper specifically discusses the cooling applications of nanofluids.

4.1 Cooling applications

The cooling applications of nanofluids include Crystal Silicon Mirror Cooling, Electronics cooling, Vehicle cooling, Transformer cooling, Space and Nuclear systems cooling, Defense applications and so on. Nano electronics refer to the use of nano technology on electronic components, especially transistors. Although the term nano technology is generally defined as utilizing technology less than 100nm in size, nano electronics often refer to transistor devices that are so small that inter atomic interactions and quantum mechanical properties need to be studied extensively. The aim of nano electronics is to process, transmit and store information by taking advantage of properties of matter that are distinctly different from macroscopic properties. The last few decades has seen an exponential growth in micro chip capabilities due to primarily a decrease in the minimum feature sizes. Nano Electronics thus needs to be understood as a general field of research aimed at developing an understanding of the phenomenal characteristics of nanometer sized objects with the aim of exploiting them for information processing purposes.

An ultrahigh-performance chip cooling device called the nanofluid oscillating heat pipe (OHP) was developed (Ma et al., 2006). They proposed the novel concept of combined nanofluids and OHPs for the breakthrough of chip cooling as they established that an OHP with water-based nanofluids containing Al_2O_3 nanoparticles has the ability to remove heat in excess of $1000W/cm^2$. This innovative and interesting discovery will surely advance the state of the art in nanofluid applications and accelerate development of a highly efficient cooling device for ultra high-heat-flux electronic systems.

It is quite interesting to note that nanoparticles can be dispersed not only in coolants and engine oils, but also in transmission fluids, gear oils, and other fluids and lubricants. Actually nanofluids provide better overall thermal management and better lubrication. The results from the first application of nanofluid research in cooling a real-world automatic power transmission system on the experimental platform of the real rotary blade coupling (RBC) of a power transmission system of a real-time four-wheel-drive vehicle show that CuO nanofluids have the lowest temperature distribution at both high and low rotating speed and accordingly the best heat transfer effect (Tzeng et al., 2005). Really, it shows a real-world application of nanofluids and as a consequence represents a gaint step forward for industrial applications of nanofluids.

The power generation industry is interested in transformer cooling application of nanofluids for reducing transformer size and weight. The ever-growing demand for greater electricity production will require upgrades of most transformers at some point in the near future at a potential crest of millions of dollars in hardware retrofits. If the heat transfer capability of existing transformers can be increased, many of the upgrades may not be necessary. It was demonstrated that the heat transfer properties of transfer oils can be improved by using nanoparticle additives specially, nanofluid-based transformer oil is likely to be the next-generation cooling fluid in transformers (Xuan and Li , 2000). The first key element in nanofluid technology which is the uniform dispersion of non agglomerated nanoparticles is still

challenging for new combination of nanoparticle-based fluid and more focus is needed on the study of dynamic interactions between nanoparticles and liquid molecules and interface structure and chemistry.

There are a number of military devices and systems such as high-powered military electronics, military vehicle components, radars and lasers which require high-heat-flux cooling. In reality, cooling with conventional heat transfer fluid is difficult for such conditions. Some specific examples of potential military applications include power electronics and directed-energy weapons cooling. Nanofluids provide advanced cooling technology for military vehicles, submarines and high-power laser diodes. Nanofluid research for defense application considers multifunctional nanofluids with added thermal energy storage or energy harvesting through chemical reactions.

The novel projected applications of nanofluids include sensors and diagnostics that instantly detect chemical warfare agent in water or water or food borne contamination; biomedical applications include cooling medical devices, deleting unhealthy substances in the blood, cancer treatment or drug delivery; and development of advanced technologies such as advanced vapour compression refrigeration systems. It is clear that nanofluids will be increasingly important for high-value added niche applications as well as for high- volume applications.

4.2 Biomedical Applications

Nanofluids can be formulated for a variety of uses for faster cooling. Nanofluids are now being developed for medical applications, including cancer therapy. As traditional cancer treatment methods have significant side effects, Iron-based nanoparticles can be used as delivery vehicles for drugs or radiation without damaging nearby healthy tissue by guiding the particles up the bloodstream to a tumour with magnets. Moreover, nanofluids could be used for safer surgery by cooling around the surgical region, thereby enhancing a patient's chance of survival and reducing the risk of organ damage. In contrast to cooling, nanofluids could be used to produce higher temperatures around tumours; to kill cancerous cells without affecting nearby healthy cells (Jordan et al.,1999).

4.3 Nano drug Delivery

Most bio-MEMS studies were done in academia in the 1990s, while recently commercialization of such devices have started. Examples include an electronically activated drug delivery microchip; a controlled delivery system via integration of silicon and electroactive polymer technologies; a MEMS-based DNA sequencer developed by Cepheid; and arrays of in-plane and out-of-plane hollow micro-needles for dermal/transdermal drug delivery as well as nanomedicine applications of nanogels or gold-coated nanoparticles An objective of the advanced endeavors in developing integrated micro- or nano-drug delivery systems is the interest in easily monitoring and controlling target-cell responses to pharmaceutical stimuli, to understand biological cell activities, or to enable drug development processes.

While conventional drug delivery is characterized by the ''high-and-low" phenomenon, microdevices facilitate precise drug delivery by both implanted and transdermal techniques. This means that when a drug is dispensed conventionally, drug concentration in the blood will

increase, peak and then drop as the drug is metabolized, and the cycle is repeated for each drug dose. Employing nano-drug delivery (ND) systems, controlled drug release takes place over an extended period of time. Thus, the desired drug concentration will be sustained within the therapeutic window as required.

A nanodrug-supply system, that is, a bio-MEMS was introduced by Kleinstreuer. (Kleinstreuer et al., 2008). Their principal concern were the conditions for delivering uniform concentrations at the microchannel exit of the supplied nano-drugs. A heat flux which depends on the levels of nano-fluid and purging fluid velocity was added to ascertain that drug delivery to the living cells occurs at an optimal temperature, that is, . The added wall heat flux had also a positive influence on drug-concentration uniformity. In general, the nano-drug concentration uniformity is affected by channel length, particle diameter and the Reynolds number of both the nanofluid supply and main microchannels. Since the transport mechanisms are dependent on convection— diffusion, longer channels, smaller particle diameters as well as lower Reynolds numbers are desirable for best, that is, uniform drug delivery.

4.4 Cancer Treatment

There is a new initiative which takes advantage of several properties of certain nanofluids to use in cancer imaging and drug delivery. This initiative involves the use of iron-based nanoparticles as delivery vehicles for drugs or radiation in cancer patients. Magnetic nanofluids are to be used to guide the particles up the bloodstream to a tumor with magnets. It will allow doctors to deliver high local doses of drugs or radiation without damaging nearby healthy tissue, which is a significant side effect of traditional cancer treatment methods. In addition, magnetic nanoparticles are more adhesive to tumor cells than non-malignant cells and they absorb much more power than microparticles in alternating current magnetic fields tolerable in humans; they make excellent candidates for cancer therapy.

Magnetic nanoparticles are used because as compared to other metal-type nanoparticles, these provide a characteristic for handling and manipulation of the nanofluid by magnetic force. This combination of targeted delivery and controlled release will also decrease the likelihood of systemic toxicity since the drug is encapsulated and biologically unavailable during transit in systemic circulation. The nanofluid containing magnetic nanoparticles also acts as a super-paramagnetic fluid which in an alternating electromagnetic field absorbs energy producing a controllable hyperthermia. By enhancing the chemotherapeutic efficacy, the hyperthermia is able to produce a preferential radiation effect on malignant cells.
There are numerous biomedical applications that involve nanofluids such as magnetic cell separation, drug delivery, hyperthermia, and contrast enhancement in magnetic resonance imaging. Depending on the specific application, there are different chemical syntheses developed for various types of magnetic nanofluids that allow for the careful tailoring of their properties for different requirements in applications. Surface coating of nanoparticles and the colloidal stability of biocompatible water-based magnetic fluids are the two particularly important factors that affect successful application

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Nanofluids could be applied to almost any disease treatment techniques by reengineering the nanoparticles' properties. In their study, the nanoparticles were laced with the drug docetaxel to

be dissolved in the cells' internal fluids, releasing the anticancer drug at a predetermined rate. The nanoparticles contain targeting molecules called aptamers which recognize the surface molecules on cancer cells preventing the nanoparticles from attacking other cells. In order to prevent the nanoparticles from being destroyed by macrophages—cells that guard against foreign substances entering our bodies—the nanoparticles also have polyethylene glycol molecules. The nanoparticles are excellent drug-delivery vehicles because they are so small that living cells absorb them when they arrive at the cells' surface.

For most biomedical uses the magnetic nanoparticles should be below 15 nm in size and stably dispersed in water. A potential magnetic nanofluid that could be used for biomedical applications is one composed of FePt nanoparticles. This FePt nanofluid possesses an intrinsic chemical stability and a higher saturation magnetization making it ideal for biomedical applications. However, before magnetic nanofluids can be used as drug delivery systems, more research must be conducted on the nanoparticles containing the actual drugs and the release mechanism.

4.5 Cryopreservation

Conventional cryopreservation protocols for slow-freezing or vitrification involve cell injury due to ice formation/cell dehydration or toxicity of high cryoprotectant (CPA) concentrations, respectively. a novel cryopreservation technique to achieve ultra-fast cooling rates using a quartz micro-capillary (QMC) was developed.The QMC enabled vitrification of murine embryonic stem (ES) cells using an intracellular cryoprotectant concentration in the range used for slowing freezing. More than 70% of the murine ES cells post-vitrification attached with respect to non-frozen control cells, and the proliferation rates of the two groups were alike. Preservation of undifferentiated properties of the pluripotent murine ES cells post-vitrification cryopreservation was verified using three different types of assays. These results indicate that vitrification at a low concentration of intracellular cryoprotectants is a viable and effective approach for the cryopreservation of murine embryonic stem cells.

4.6 Nanocryosurgery

Cryosurgery is a procedure that uses freezing to destroy undesired tissues. This therapy is becoming popular because of its important clinical advantages. Although it still cannot be regarded as a routine method of cancer treatment, cryosurgery is quickly becoming as an alternative to traditional therapies.

Simulations were performed on the combined phase change bioheat transfer problems in a single cell level and its surrounding tissues, to explicate the difference of transient temperature response between conventional cyrosugery and nanocyrosurgery (Yan and Liu, 2008). According to theoretical interpretation and existing experimental measurements, intentional loading of nanoparticles with high thermal conductivity into the target tissues can reduce the final temperature, increase the maximum freezing rate, and enlarge the ice volume obtained in the absence of nanoparticles. Additionally, introduction of nanoparticle enhanced freezing could also make conventional cyrosurgery more flexible in many aspects such as artificially interfering in

the size, shape, image and direction of iceball formation. The concepts of nanocyrosurgery may offer new opportunities for future tumor treatment.

With respect to the choice of particle for enhancing freezing, magnetite and diamond are perhaps the most popular and appropriate because of their good biological compatibility. Particle sizes less than 10μm are sufficiently small to start permitting effective delivery to the site of the tumor, either via encapsulation in a larger moiety or suspension in a carrier fluid. Introduction of nanoparticles into the target via a nanofluid would effectively increase the nucleation rate at a high temperature threshold.

4.7 Magnetic Nanofluids

Magnetic nanofluids are prominent materials in engineering as well as biomedical applications. In medical applications, for example, ferrofluid provides new cancer treatment techniques by employing iron based nanoparticles as delivery vehicles for drugs or radiation. Our group synthesizes magnetic nanofluids by decorating carbon based nanostructures with magnetic materials. The thermal conductivity of these nanofluids has been measured with magnetic field and without magnetic field. With magnetic field the nanofluid shows more enhancements in thermal conductivity than that without magnetic field. This anomalous enhancement is due to the alignment of magnetic dipole in the direction of magnetic field. When magnetic dipoles will align in the direction of magnetic field, there will be thermal continuity between the particles, which increases the thermal conductivity.

In the medical field nanofluids are sometimes referred to as nano-scale colloidal solutions. Nanofluids is used biosensors, which is one medical application of nanofluids. The two biggest applications to date are in cancer research and optofluid control of fluid motion. Once again, many of these concepts are still in the laboratory stage, but the initial results are promising.

For cancer research there are two (related) major areas: nanovectors and nanofluid hyperthermia (i.e. heat treatment). With nanovectors, the particles can range from magnetic materials (e.g. iron-oxide based particles for MRI) to liposomes. Liposomes are ideally designed with drug or gene therapy imbedded in them and a surface which will be selectively attracted to the area of interest. For imaging applications, the goal is to image early stage diseases. Again, particles designed such that they will selectively image the area of interest (cancer cells, viral particles, antherosclerosis, etc.). Quantum dots when dispersed in a fluid (i.e. in medical imaging), can also be considered an application of nanofluids since they range from 1-10 nm in size.

The other important area which has seen a lot of interest in recent years is hyperthermia treatments using in-vitro nanoparticles (i.e. nanofluids). The basic idea again requires the particles to selective attach to diseased regions or cells. Next, the region is irradiated – usually with a magnetic field. Since magnetic nanoparticles are highly absorbing compared to the surrounding fluid, the nanoparticles can heat up enough cause severe damage (thermoablation) to the adjacent cells. This is a simple idea, but controlling the process enough to leave healthy tissues unharmed is a challenge.

Another exiting idea that employs nanoparticles is optofluidic or magnetic control of fluid flow. Among other applications, this shows potential for biomedical applications - to assist the above

selective treatments or to control microfluidic mixing/separation. In this area of research, nanoparticles are again the absorbing medium which becomes input energy for driving fluid flow. For example, through a process of evaporation and condensation, researchers have been able to drive flow. Optical tweezers are another way of controlling nanoparticles in solution. Wang et al. demonstrated that particles can be controlled finely enough to be rotated about their axis . Alternatively, magnetic fields can be used to control magnetic nanofluids. In a magnetic flow scheme, pH controls influences the magnetophoretic velocity of smart‘ magnetic nanoparticles – which is an intriguing way to control mixing/separation.

In medical applications, nanofluids (even though they may not be called such) are finding a myriad of applications. Based on the publications, it is safe to say that the potential market and the growth of applications in this field matches or surpasses any conductive/convective heat transfer nanofluid applications.

5. Conclusion

One of the goals of theoretical research on nanofluids is to develop a theory of nanofluids to explain how nanoparticles change the thermal properties of nanofluids. A theory of nanofluids would also provide a theoretical foundation for physics and chemistry based predictive models.

Numerous studies conducted on nanofluids have made scientific breakthrough not only in discovering unexpected thermal properties of nanofluids, but also in proposing new mechanisms behind the enhanced thermal properties of nanofluids and thus identifying unusual opportunities to develop them as next generation coolants for computers and safe coolants for nuclear reactors. Applied research in nanofluids has demonstrated in the laboratory that nanoparticles can be used to enhance the thermal conductivity and heat transfer performance of conventional heat transfer fluids. Some researchers took the concept one step into practical applications and demonstrated the ability of nanofluids to improve the performance of real world devices and systems such as automatic transmissions. Thus, nanofluid research has made the initial transition from our laboratory to industrial research laboratories. This important work has provided guidance as to the right direction, the first step in the development of commercial nanofluid technology. With continued collaboration between basic and applied nanofluid researchers in academia and industry on thermal properties, performance, theory, mechanisms, modeling, development and eventual commercialization of nanofluids, nanofluid research is expected to bring breakthroughs in nanotechnology-based cooling technology and have a strong impact on a wide range of engineering and biomedical applications. In future, promising nanofluids should be studied not only under real- world conditions of use, but also over a longer period of time. Nanofluids will be in number one position for the contribution of the humanity to the newer horizons. Concluding, we can remark that surely, coming days are for the nanofluids.

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Development of mitochondria targeted antioxidants as mitochondrial medicines

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Abstract

Mitochondrial research is presently one of the fastest growing disciplines in biomedicine. In mitochondria, reactive oxygen species (ROS) are generated as undesirable side products of the oxidative energy metabolism. Thus, Mitochondria are targets of their own oxidant by-products leading to the mitochondrial dysfunction. It has been hypothesized that major factor in the dysfunction of mitochondria results from the defects in oxidative phosphorylation (OXPHOS) that results in the stimulation of the mitochondrial production of ROS and damage to mitochondrial DNA (mt DNA). The dysfunction of the mitochondria has been proposed to be the main reason behind many diseases including diabetes mellitus, heart disease and neurodegeneration, as well as in the aging process. Tissues with high metabolism seem to be particularly vulnerable to mitochondrial dysfunction. Therefore, protection of mitochondria from oxidative stress is of prime importance to all organisms. This can be done by enrichment of the mitochondria with antioxidants which has been employed successfully in recent *in vitro* and *in vivo* experiments. Based on the recent exciting developments in mitochondrial research, increasing pharmacological efforts have been made leading to the emergence of 'Mitochondrial Medicine'. The targeted and carrier-based delivery of drugs and DNA to mitochondria hardly constitutes a field of research on its own yet and is still in its infancy.

Key words: Reactive oxygen species; oxidative stress; antioxidants; free radicals

1. Introduction

In cell biology, a mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells (Henze & Martin, 2003). Mitochondria are sometimes described as "cellular power plants" because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy (Campbell et al., 2006). The most prominent roles of mitochondria are to produce ATP (i.e., phosphorylation of ADP) through respiration, and to regulate cellular metabolism (Voet et al., 2006). Mitochondria play a vital role in cellular homeostasis. They house the OXPHOS machinery, and multiple metabolic pathways, such as β-oxidation of fatty acids and the TCA and urea cycles. In addition, mitochondria have important biosynthetic activities, control intracellular Ca^{2+} metabolism and signaling, regulate thermogenesis, generate cellular reactive oxygen species (ROS) and serve as the gatekeeper of the cell for programmed cell death (apoptosis) (Scheffler, 2001). Again, mitochondria are primarily responsible for meeting the enormous energy demands of the 'fight and flight response' in vital tissues, by oxidizing the large amounts of substrates that are made available by stress hormone- induced mobilization from energy storages (Manoli, et al., 2007).

A rapidly expanding body of literature also suggests that mitochondrial dysfunctions play pivotal roles in many human disorders, including neurodegenerative diseases, ischaemia-reperfusion injury, aging and inflammatory damage (Ames et al., 1993; Wallace, 1995). Mitochondrial dysfunction is inherent in a variety of human disorders from the classical

mitochondrial diseases arising from mitochondrial DNA mutations (encephalomyopathies) to those involving mitochondrial signaling pathways to the rest of the cell, modulated by organellar dynamics and culminating in programmed cell death. Beyond minding the cell's energy status, mitochondria are major cellular sources (and targets) of free radicals and are effectors of the intrinsic apoptotic pathway due to the release of signaling molecules that activate and trigger specific caspase cascades. The electrontransfer protein cytochrome c has been recognized over 20 years ago as a signaling molecule released from mitochondria, thus changing the view of these organelles from their energy-transducing functions to a regulatory role in cell death pathways. Hence, it is not surprising that treatment of mitochondrial dysfunction and development of mitochondrion- targeted therapeutics have become a primary focus of scientific research.

2. Mitochondrial damage induced by oxidative stress

Damages to mitochondrial macromolecules are exaggerated during oxidative stress as is observed especially in pathological conditions. The pathological symptoms may result either from reactive oxygen species (ROS) and reactive nitrogen species (RNS) mediated damage of macromolecules or from the changes in the gene expression. The generated ROS have very short life span and can readily react with the macromolecules such as lipids, proteins and nucleic acids of mitochondria.

The excessive generation of free radicals leads to peroxidative changes that ultimately result in enhanced lipid peroxidation in the mitochondria. Cardiolipin, an important phospholipid that serves as a cofactor for a number of critical mitochondrial transport proteins and retains cytochrome c at inner mitochondrial membrane through electrostatic interaction, declines due to oxidative damage. Loss of cardiolipin, coupled with oxidation of critical thiol groups in key proteins, may adversely affect transport of substrates and cytochrome c oxidase activity that is necessary for mitochondrial function leading to apoptotic cell death (Ott et al., 2002). ROS-induced oxidative modification of many enzyme proteins inside mitochondria results in structural alteration and their functional inactivation. It has been demonstrated that under oxidative stress conditions the oxidized proteins instead of undergoing proteolytic digestion, aggregated by cross liking with one another and affect the normal cellular functions (Babusíková et al., 2004).

mtDNA is highly susceptible to oxidative damage probably due to the (1) close proximity to the site of ROS/RNS production for being inside mitochondria, and that superoxide ($O_2^{\cdot -}$) generated inside mitochondria is not permeable to cytosol (2) mtDNA lacks histone proteins (which protects nuclear DNA from oxidative damage); and (3) mitochondrial polymerases lack specificity for base excision repair, which is the major pathway eliminating oxidative DNA base lesions. Oxidative damage to mt DNA such as strand breaks and base modifications can occur either directly from ROS or from ROS derived lipid-hydroperoxide.

3. Mitochondrial medicine

The current research scenario on the mitochondrial research has been resulted in the emergence of ‘Mitochondrial Medicine’ as a new field of biomedical research (Murphy and Smith, 2000). However, research on the designing and evaluation of mitochondria-specific drugs, carriers or delivery systems which are collectively called as ‘Mitochondrial Pharmaceutics’ is still in its infancy. The accumulation of somatic mutations in the mitochondrial genome has been suggested to be involved in aging, age-related neurodegenerative diseases as well as in cancer. Also, an increasing number of xenobiotics and pharmaceutics are being recognized to manifest their toxicity by interfering with mitochondrial functions (Wallace and Starkov, 2000). In conclusion, the mitochondrial

targeted drugs could effectively emerge as unique solutions for a large variety of future cytoprotective and cytotoxic therapies.

3.1 Selection of oral antioxidants as mitochondrial medicines

Targeting of biologically active molecules to mitochondria in living cells will open up avenues for manipulating mitochondrial functions. The delivery of antioxidants may protect mitochondria from oxidative stress caused by a variety of insults; perhaps even contribute to slowing down the natural aging process. Attempts to achieve cell protection using antioxidants have already successfully been undertaken, many of them utilizing the avid reactivity of fullerene compounds with free radicals. The increase of mitochondrial concentrations of antioxidant drugs by selective targeting antioxidants to mitochondria in living cells should therefore be an effective therapy for a wide range of human diseases (Galley, 2010).

Large number of antioxidants have shown protective effects against aging and other degenerative diseases in which mitochondria plays the most important role. Vitamin A/Carotenoids are longevity determinants (Cutler, 1984). The effects of postnatal administration of an excess of vitamin A on DNA, protein and metabolism and pyridine nucleotide-linked dehydrogenases in various organs including the brain have been studied by Shukla et al. (1984). Craft et al. (2004) reported an age-related reduction in carotenoids, xanthophylls (oxygenated carotenoids), retinol and tocopherol in the frontal lobe cortex of the human brain, which is vulnerable in Alzheimer's disease, suggesting a role of these vitamins in the brain. Seidman (2000) inferred from his study using rats that treatment with antioxidants such as vitamins C and E would reduce the degree of age-related hearing loss.

Bourre (1991) reported that vitamin E protected membrane PUFA against radical peroxidation during cerebral aging, especially in cerebral capillaries and microvessels. Vitamin E protects against nerve terminal dysfunction caused by oxidative stress. Dietary vitamin E plus exercise training corrected the age-related deficit in SOD, GPx and CAT in rat cerebral cortex and hippocampus (Devi and Kiran, 2004). Vitamin E reduced the levels of MDA and lipofuscin and increased the activities of SOD and CAT in the brains of *D* -galactose- induced aging mice (Ling, 2004).

Recently, a great deal of attention has been focused on the antioxidant activities of α-lipoic acid (Packer, 1995; Bilska and Włodek, 2005). α -lipoic acid is essential to cell energy metabolism, is a cofactor at entry to Krebs cycle, displays anti-oxidant effects by increasing the GPx activity and reducing oxidative and regulates calcium homeostasis (Bilska and Włodek, 2005). Indeed several studies have shown that α -lipoic acid exerts multiple pharmacological actions able to prevent nerve degeneration in experimental in vitro models of Parkinson disease (Bharath et al., 2002), and Alzheimer diseases (Mohmmad Abdul and Butterfield, 2007). It also reduces damage from ischemia–reperfusion in animal models (Freisleben, 2000). Recent studies showed that it can decrease the age related decline of antioxidant status and cellular energy status and decrease the oxidative stress in various animal models (Savitha et al., 2007; Tamilselvan et al., 2007).

Similarly of acetyl- L -carnitine (ALCAR) is another potent antiaging drug which has been studied in detail (Kumaran et al., 2004; Savitha et al., 2007; Tamilselvan et al., 2007). Fraschini et al. (1991) suggested that the anti-aging action of ALCAR might be attributed to increased melatonin synthesis. Carnitine may increase melatonin secretion, reduce lipid peroxidation and improve the antioxidant status. Again, natural antioxidants such as *Centella asiatica*, grape seed etc have been shown to have potent antioxidant activities which protect against oxidative stress induced damages associated with aging (Balu et al., 2005; Subathra et al., 2005) Similarly, polyphenols, isoflavones, ginsenosides and flavonoids, extracted from

medicinal plants, have antioxidant function and are proved to have protective effects on mitochondrial function. Green tea polyphenols are believed to be a strong antioxidant against $HO^{\cdot}$, $NO^{\cdot}$ and lipid oxidation (Nanjo et al., 1996; Panickar et al., 2009).

The mitochondria targeted version of vitamin E protected mitochondria from oxidative damage induced by iron/ascorbate far more effectively than vitamin E itself, as measured by the level of both, lipid peroxidation (thiobarbituric acid reactive species) and protein damage (protein carbonyls) (Smith et al., 1999). Some antioxidants such as vitamin E, ubquinol and N-acetyl cysteine have been shown to decrease mitochondrial oxidative damage, but because these compounds were not accumulated within mitochondria their effectiveness was limited (Matthews et al., 1998). Thus, antioxidants which can stay long inside mitochondria will be more effective. Recently, mitochondrially targeted versions of vitamin E (MitoE) and ubiquinone (MitoQ) were synthesized and characterized and found that they can selectively accumulate inside mitochondria and protect it from oxidative damage far more effectively than vitamin E and ubiquinone itself (Smith et al., 2008). Co-enzyme Q10 (CoQ10) and L-acetyl-carnitine can be considered to be safe adjunct to standard therapies in cardiovascular and neurological diseases (Fosslien, 2003). Carvediol, cardiovascular drug has been proved to be effective in heart failure probably mediated through its potential antioxidant and antiapoptotic activities (Feuerstein et al., 1998). Recently studies have been doing for the development of effective mitochondria-targeted antioxidants composed entirely of natural constituents. Lyamzaev et al. (2011) synthesized novel mitochondria-targeted antioxidants containing plant electron carrier and antioxidant plastoquinone conjugated by nonyloxycarbonylmethyl residue with berberine or palmatine, penetrating cations of plant origin. Two free radical scavengers, 4-hydroxy-2,2,6,6-tetramethylpiperidin-N-oxide (TEMPOL) and Salen-Mn(III) complex of o-vanillin (EUK-134) have been successfully synthesized and partially tested in term of their antioxidant and antiapoptotic properties (Dessolin *et al*., 2002).

Recent reports showed that a palladium alpha lipoic acid complex (POLY MVA) had effectively prevented age related oxidative stress in the mitochondria of aged Wistar rats by enhancing mitochondrial antioxidant enzymes (Sudheesh et al., 2010) and mitochondrial dehydrogenases and respiratory chain complexes (Sudheesh et al., 2009). Similarly, the well known medicinal mushroom, *Ganoderma lucidum* which is popularly known as Reshi or Lingzhi and has been traditionally used as a popular folk medicine for the promotion of health in the Orient reported to significantly ameliorate the oxidative stress induced decline in the mitochondrial antioxidant status and the activities of respiratory chain complexes and mitochondrial dehydrogenases in the heart and brain of aged rats (Ajith et al., 2009; Sudheesh et al., 2009, 2010). Moreover, this mushroom is also reported to prevent the heart and liver mitochondria from oxidative damage by chemicals such as Isoproterenol and acetaminophen. The mechanism had been found to be through the enhancement of the mitochondrial antioxidant status, respiratory chain complexes, mitochondrial dehydrogenases and mitochondrial membrane potential and by direct scavenging of mitochondrial ROS produced during oxidative stress (Sudheesh et al., 2011, 2012).

This suggests that therapies designed to interfere with oxidative stress could be prevent from mitochondrial damages associated with oxidative stress and aging. Though many of the drugs used in the treatment of cardiovascular diseases, mainly statins are proved to be antioxidants, data from well-designed randomized trials to issue the general recommendation for people to take antioxidant supplements in order to prevent the oxidative stress induced disease is insufficient (Ajith et al., 2006; Ajith et al., 2008). Thus, the area of sub-cellular, i.e. mitochondria-specific delivery of drugs is still in its infancy.

3.2 New developments in the mitochondria targeted antioxidants

However, despite the clinical importance of mitochondrial oxidative damage, antioxidants have been of limited therapeutic success. This may be because the antioxidants are not selectively taken up by mitochondria, but instead are dispersed throughout the body. To address this unmet need, a series of mitochondria-targeted antioxidants have been developed over the past few years that are selectively concentrated within mitochondria *in vivo*. The accumulation of an antioxidant at the site where it is needed most has been shown to improve the outcome in a large number of animal models of diseases that involve mitochondrial oxidative damage. Mitochondria-targeted antioxidants have also been developed as pharmaceuticals and have been shown to be safe and effective in human clinical trials. Therefore, the mitochondria-targeted antioxidants are a new class of pharmaceuticals that can be used in a wide range of human pathologies for which current therapies are of limited efficacy.

The targeting of small antioxidant molecules to mitochondria *in vivo* have been effectively performed by two common methods such as conjugation to lipophilic cations (Murphy and Smith, 2007) or incorporation into mitochondria-targeted peptides (Horton *et al.*, 2008). Triphenylphosphonium (TPP) is the best characterized and most widely used lipophilic cation for delivery of antioxidants to mitochondria and there are a wide range of antioxidants that have been targeted to mitochondria by conjugation to the TPP lipophilic cation. Vitamin E (Smith *et al.*, 1999), ebselen (Filipovska *et al.*, 2005), lipoic acid (Brown *et al.*, 2007), plastoquinone (Skulachev *et al.*, 2009), nitroxides (Trnka *et al.*, 2008), and nitrones (Murphy *et al.*, 2003) are some of them. The best characterized antioxidant targeted to mitochondria by conjugation to the TPP cation is MitoQ. MitoQ has been shown to be protective in a large number of cell models of mitochondrial oxidative stress (reviewed in Murphy and Smith, 2007) such as against heart dysfunction (Neuzil *et al.*, 2007), liver damage (Lowes *et al.*, 2008, type I diabetes (Chacko *et al.*, 2010), cocaine induced cardiac dysfunction (Vergeade *et al.*, 2010) etc. Together these findings show that MitoQ is protective against pathological changes in a number of animal models of mitochondrial oxidative damage that are relevant to human diseases.

An alternative approach to targeting antioxidants to mitochondria is through the use of small, positively charged peptides called Szeto-Schiller (SS)-peptides (Zhao *et al.*, 2004). SS-peptides comprise four alternating aromatic/basic amino acids with a D-amino acid in the first or second position along with amidation of the C-terminus to make them more resistant to degradation (Szeto, 2006). The SS-peptides have three positive charges at physiological pH, and studies with isolated cells showed their rapid uptake through the plasma membrane and accumulation by mitochondria, where they bind to the inner membrane (Zhao *et al.*, 2003). Some of the SS-peptides have intrinsic antioxidant activity; for example in the SS-31 peptide, the most effective tested to date, the antioxidant activity is due to a dimethyltyrosine residue which is thought to act through its phenolic moiety (Szeto, 2006) while similar peptides without this residue were not protective. These SS-peptides are protective against oxidative stress in isolated mitochondria and in cell models of disease in including reperfusion injury, diabetes etc (Zhao *et al.*, 2004; Manczak *et al.*, 2010; Whiteman *et al.*, 2008). Therefore the SS-peptides can be delivered *in vivo* by i.p. or i.v. administration and are then protective against mitochondrial damage in a wide range of animal models.

4. Conclusions and Future Challenges

Despite their central role as powerhouse of cell and their inevitable participation in all the major pathways of cell, mitochondrial targeting drugs have been a neglected till date (Murphy, 2009). This is due to the difficulty in concentrating drugs towards this organelle in

vivo. The discovery of new and modified antioxidants which can accumulate themselves inside mitochondria will be the next important area of mitochondrial research. The field of targeting therapeutic molecules to mitochondria is just beginning, but as mitochondrial damage contributes to so many diseases it is likely that further therapeutic compounds will be developed and applied to important human pathologies. The available references in this area clearly shows that clinical complications such as cardiovascular diseases, hepatic diseases, aging, cancer, degenerative diseases etc will be the important candidates for treatment by this approach in the coming years. However, the detailed *in vivo* and *in vitro* studies are needed to develop new and modified therapeutic antioxidants which can effectively accumulate inside mitochondria and there by ameliorate various diseases and this will surely change the face of biomedical research and drug discovery

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