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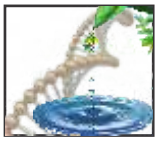

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Halophiles and their Important Enzymes Used for Biotechnology Application

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ABSTRACT: *Halophilic bacteria can grow in hypersaline environments like salt mines, solar salterns, hydrothermal, marshy lagoons and hypersaline. They have ability to adapt salt stress conditions by accumulating different water soluble organic molecules such as ectoine, glutamate, glycine betaine and proline. Mostly Haloarchaea can flourish in hypersaline environments by accumulating potassium chloride ions to maintain their internal environments. Bacterial proteins confer osmoregulation, toxic heavy metal resistance and enzymes that expand the nutritional ability of the cell. Metaproteomics (environmental proteomics study deals with all the proteins estimated directly from environmental samples (soil, water, plant or animal tissues). The field of environmental biotechnology has been widely extended by the recent advances in metagenomics levels. It also extended the post genomic area of research.*

Key Words: *Halophiles, Enzymes, osmo-adaptative mechanisms*

INTRODUCTION

Halophiles are salt loving organisms and are found in saline conditions. These organisms can be classified in to slight, moderate and extreme halophilic organisms (Shivanand and Mugeraya, 2011). Their classification depends on the requirement of sodium. Hypersaline environments are widely found in coastal, deep sea, arid and salt mines. Halophilic microorganisms include a variety of phototrophic, heterotrophic and photosynthetic organisms (DasSarma and DasSarma 2012).

The ability of microorganism to adapt the changes in the osmolality of the external milieu is of fundamental importance for growth and survival, and thus prokaryotic cells have evolved a number of osmoadaptative mechanisms to cope with elevated osmolality. One hundred bacterial isolates were isolated from soil from Egypt and screened for salt tolerance (Fernandez-Aunión et al., 2010).

Salt is required for all forms of life. Halophiles are unique by their requirement high salt conditions for growth. They can also be distinguished by their salt requirement. Some grow optimally at 0.2-0.85 M sodium chloride. Moderate halophiles grow at 0.85- 3.4 M and extreme halophiles grow up to 3.4-5.1 M (Jamadar et al.,2016). Halotolerant organisms can grow in high salinity or in the absence of high salt concentrations. Hypersaline conditions are lethal to most cells because of the loss of high water content from the cell. Halophiles accumulate high salt concentrations in their cytoplasm to prevent any loss of cellular water. Cell volume is maintained by isosmotic balance. The osmolytes accumulate in halophiles are sugars, polyols and different amino acids (Ventosa et al., 1998). These osmolytes do not interfere with intracellular metabolism and lack any net charge at physiological pH. Halotolerant yeast and green algae accumulate polyols, halotolerant bacteria accumulate zwitterionic species such as glycine betaine and ectoine (Abbas et al., 2006).

Solute accumulation may take place by biosynthesis or from the storage material. Haloarchea and extreme halophilic bacteria accumulate potassium chloride equal to external concentration of NaCl (Deole et al., 2013). These organisms produce acidic proteins that can function

in high salinity by remaining solvated and reducing aggregation, precipitation and denaturation.

A great biodiversity of prokaryotic halophiles exists naturally. They have been studied widely by both culturing and non culturing techniques. Phylogenetic analysis and taxonomic classification are widely used to identify the prokaryotes (Felsenstein, 1985). A method ofmultilocus sequencing typing is used to sequence the nucleotides of multiple genes. For nonculture halophiles, environmental metagenomics studies making use of 16S ribosomal ribonucleic acid genes for sequencing are used to study the diversity among halophiles (Abbas et al., 2006).

Enzymes of Halophiles

Hydrolases is a class of enzymes that is widely distributed naturally from bacteria to eukaryotes. Different screening techniques were used in recent years to isolate the important enzymes of halophiles. Several hydrolases were isolated from hyper saline conditions, including amylases, lipases and proteases and are used for biotechnological approaches (Dalmaso et al., 2015). The lipolytic (LiPBL) enzymes have advantages over other lipases, it active over a wide range of pH and temperature. The immobilized LipBL derivatives obtained and tested in regio- and enantioselective reactions, showed an excellent behavior in the production of free polyunsaturated fatty acids (PUFAs). On the other hand, the extremely halophilic bacterium, *Salicola marasensis* sp. IC10 showing lipase and protease activities, was studied for its ability to produce promising enzymes in terms of its resistance to temperature and salinity (Akhtar et al., 2008)

Marinobacter lipolyticus SM19, an important intracellular enzyme produced by halophilic bacterium was isolated and characterized. LipBL Lipolytic enzyme was assigned to the family VIII of lipolytic enzymes. Its expression was measured in E.coli. The molecular weight of LipBL (lipolytic enzyme) protein is 45.3 kDa and it is 404 amino acids long. LipBL was purified and biochemically characterized. The temperature for its maximal activity was 80 qC and the pH optimum determined at 25 qC was pH 7.0, showing optimal activity without sodium chloride, while maintaining 20% activity in a wide range of NaCl concentrations (AnbuRajan et al., 2008).

Proteases

The most important group of enzymes that are used in industry are proteases. Food industry and in washing detergents, these enzymes has a worldwide commercial importance. Halophilic proteases are good for industry because of their stability and properties. Haloprotease CP1 has been isolated from the moderately halophilic bacterium *Pseudoalteromonas ruthenica*, it is an extracellular protease. This enzyme was purified by using ion exchange gel filtration chromatography. The protease was also extracted from Halobacillus karajensis strain MA-2 and characterized. This enzyme belongs to the class serine metalloproteases. These findings suggested that this enzyme has great biotechnological application and potential during alkaline conditions (Moreno et al., 2009).

Amylase

Amylases are considered important enzymes in industry. They have a wide spread applications in starch sachharification, clinical and analytical chemistry. Many D-amylases are were purified from moderate halophiles e.g Nesterenkonia sp. strain F. the amylase extracted from Nesterenkonia sp. strain F have molecular weight of 110 kDa, it was determined by using SDS-PAGE. This enzyme show maximum activity at pH 7-7.5. The temperature at which it show best activity is 45 °C. The extracted enzyme is highly stable under the saline conditions ranging from 0 to 4%, its activity is not affected by the presence of Ca^{2+}, Rb^{+}, Li^{+}, Cs^{+}, Mg^{2+} and Hg^{2+} but its activity is stop or effected by Fe^{2+}, Cu^{2+}, Zn^{2+} and Al^{2+}. These amylases can also stop activity when effected to EDTA.

Another D-amylases was extracted and purified from halophilic *Thalassobacillus* sp. LY18 .This enzyme showed a molecular mass of 31 kDa and its optimal enzyme activity was found to be at 70 qC (Pinar et al., 2014).

Lipolytic and Proteolytic Enzymes

Some hydrolases are also extracted from *Salicola marasensis* IC10, it produces an extracellular protease. These bacteria are extreme

halophiles, intracellular lipase LipL was also purified from this bacterium. This enzyme is active in presence of different compounds as substrates: p-nitrophenyl butyrate, p-nitrophenylvalerate, p-nitrophenylcaprilate and p-nitrophenyldecanoate as well as 4-methylumbelliferone and the enzyme production is maximal at the end of the exponential phase (Hu et al., 2005).

The characterization of the intracellular fraction of *Salicola* sp. IC10 during growth was performed, finding the optimal conditions at pH 8.0, 40 qC and a medium with 15%±20% (w/v) NaCl. Thus, there is a correlation between the optimal conditions for cultivation of the strain and the maximum production of the proteolytic enzyme. This protease showed the capability to effectively catalyze the hydrolysis of various proteins. The most specific substrate to the enzyme was egg albumin, followed by gelatine (97% relative activity). Therefore, we can conclude that enzymes produced by halophilic bacteria show interesting properties for use in different industries (Moreno et al., 2013).

Salt stress is one of the most significant factors that negatively affect plant growth and development. Proline has been reported to be an osmoprotectant that confers tolerance to salinity in various plant species. A cDNA for Δ1-pyrroline-5-carboxylate synthetase (P5CS), a key enzyme involved in proline biosynthesis was isolated and characterized from *E. camaldulensis*, now designated EuP5CS. The full-length EuP5CS gene has 2,944 bp containing an open reading frame of 2,142-bp that encodes for 713 amino acids. The deduced EuP5CS protein structure exhibited a high homology to the P5CS of other plant species, and was predicted to possess a glutamate 5-kinase domain at its N-terminal and a gamma-glutamyl phosphate reductase domain at its C-terminal. Semi quantitative reverse transcription polymerase chain reaction analysis revealed that the transcriptional expression level of the EuP5CS gene was considerably up-regulated by up to about 50% in response to NaCl treatments. This result indicated that EuP5CS is a salt-inducible gene and plays an important role in proline biosynthesis in *E. camaldulensis* clones subjected to salt stress (Ekchaweng et al., 2012)

CONCLUSION

Halophiles are organisms that prefer to live in highly saline conditions. Halophiles are excellent sources of enzymes that are not only salt stable but also can withstand and carry out reactions efficiently under extreme conditions. The aim of the study was to study the diversity among halophilic bacteria producing enzymes of industrial value.

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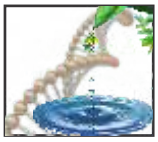

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Morpho-Physiological Variations in Pearl Millet [*Pennisetum glaucum* L. (R.Br)] in Response to Foliar Applications of NPK

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ABSTRACT: *This study was carried out to evaluate the response of Pearl millet to foliar applications of NPK fertilizer with different levels i.e. T_0 (control), T_1 (NPK 4:4:3), T_2 (NPK 8:8:6). Foliar applications of NPK fertilizer enhanced the growth and chlorophyll contents of pearl millet. Maximum increase in growth attributes were recorded in T_2 (NPK 8:8:6) while, high increase in chlorophyll and carotenoides contents of pearl millet was measured in T_1 (NPK 4:4:3) as compared to T0 (control) and T_2 (NPK 8:8:6) at both seedling and vegetative stages. Variations were also observed in chemical analysis for N and K^+ at both stages of growth under NPK applications. It was concluded that NPK are the major nutrients required for plant growth but its applications are more effective than applications in root medium.*

Key Words: *Pearl millet, NPK, morphology, physiology, chlorophyll*

INTRODUCTION

Today's major cereal crop in the arid and semi-arid regions is the Pearl millet [*Pennisetum glaucum* L. (R.Br)]. It is hardy crop that requires less water and has a short growing period which is getting more attention due to increasing evidence of less seasonal rainfall, increase in temperature, frequent occurrence of extreme weather events coupled with scanty water resources (Jakhar et al., 2006; Prakash et al., 2008). Advantage of Pearl millet over other cereal crops is that it has grown in thoseagricultural areas where annual rainfall is very less (200-500 mm) and also where daily temperature touches at 30 °C (FAO, 1996). Moreover its nutrient content and properties are equivalent and even superior to other cereals (Obalama and Manyasa, 2002).

Foliar fertilizer applications are considered to provide an added source of nutrients and also facilitate the plant to survive a wide range of stresses. Foliar fertilizer applications can increase the uptake of nutrients from soil by stimulating exudation of organic substances from the roots into the soil. The microorganisms present in the roots are stimulated by these exudates, so their activity helps to increase the availability of nutrients (Glinicki, 2010).

For plant growth and development, nitrogen (N) is an essential nutrient. To improve the yield and quality of forage, it is much essential to determine the fertilizer requirement of crop. Fertilizer's judicious and appropriate use not only increases yield but also improves quality of forage especially protein contents (Ayub et al., 2007). Potassium (K) plays a particular vital role for the translocation of nitrates to the root and shoot, increased rapid N metabolism and maintenance of water potential in contributing to the survival of crop under environmental stress conditions. Phosphorus (P) is essential for many physiological processes within the plants (Mengel and Kirkby, 2001). The essential physiological roles of P in crop growth are reviewed in considerable detail by Sinclair and Vadez (2000). Phosphorus (P) is an integral component of the biochemical compounds that make essential structural, biochemical and physiological roles in crop growth.

This study was carried out to investigate the morphological and physiological responses of

Pearl millet (variety Indo-Japan hybrid) to foliar applications of NPK fertilizer with different concentrations.

MATERIALS AND METHODS

The seeds of pearl millet variety Indo-Japan hybrid were obtained from the Agricultural seed store, Gujranwala, Pakistan. Eight seeds were sown directly into each pot (30 cm length and 27.5 cm diameter) containing 9 kg soil. Thinning was done after 8 days of germination and four plants were kept in each pot. Foliar applications of NPK fertilizer was applied 21 days after germination. The foliar applications of NPK were:

1. T_0 (control)
2. T_1 (NPK 4:4:3)
3. T_2 (NPK 8:8:6)

The design used for this study was completely randomized design (CRD) with 6 replications of each treatment. Shoot and root lengths (cm) were measured with the help of a meter rod from stem base to the top. Two plants were harvested from each pot at the seedlings stage (24 days after treatment) and vegetative stages (48 days after treatment). Plants were uprooted carefully and washed with distilled water. After harvest, shoot and root of each seedling and vegetative plant were separated and data for fresh and dry weight of shoot and root recorded. Plant samples were placed in oven at 75°C. After 4-days shoot and root dry weight (g/pot) was calculated at final harvest. Dried plant material was digested with a nitric-perchloric mixture. In shoot plus leaves ion contents of N and K^+ were determined. Total nitrogen was calculated by Kjeldhal procedure and K^+ was determined with a flame photometer. A graded series of standards (ranging from 10 to 100 mg/L) of K^+ were prepared and standard curve for K+ was drawn. K^+ contents were determined by emission spectrophotometry by determining optical density at 460 nm as described by Jackson (1962).

Chlorophyll content were extracted by using 90% acetone and the value of chlorophyll-a, chlorophyll-b and carotenoides were determined by using Arnon's (1949) equations given below

Chl-a determination = 0.0127 A663 – 0.00269 A645
Chl-b determination = 0.0029 A663 – 0.00468 A645
Carotenoides determination = OD480+ (0.114 x OD 663)-(0.638 x OD 645)/112.5

STATISTICAL ANALYSIS

Analysis of variance technique was used to carry out statistical analysis of the data (Steel and Torrie, 1980). Various treatment means were compared by Duncan's New Multiple Range (DMR) Test.

RESULTS

Foliar applications of NPK had significant effects on biomass production and some selective physiological parameters of pearl millet. Applications of foliar spray of NPK fertilizer has significant effect on various morphological attributes (Table 1 & 2). NPK increased the shoot length (cm) of pearl millet. Maximum shoot length was recorded in T_2 (NPK 8:8:6) as compared to T_0 (control) at seedling and vegetative stages. Higher concentrations of NPK had better effect in relation to maximum shoot length as compared to low concentrations of NPK as shown in Fig 1. Similarly, in case of root length, maximum root length (9cm) was observed in T_2 (NPK 8:8:6) at seedling as well as at vegetative stage. Higher concentrations of foliar NPK increased maximum root length than lower concentrations of NPK (Fig 2).

Significantly maximum shoot dry weight (g) was recorded in T_2 (NPK 8:8:6) as compared to T_0 (control) at seedling and vegetative stages (Fig 3). However, shoot dry weight of T_1 (NPK 4:4:3) recorded lower than T_2 (NPK 8:8:6) at both seedling and vegetative stages. Fig 4 showed that there was a significant difference for shoot fresh weight (g) of plants among treatments of T_0 (control), T_2 (NPK 4:4:3) and T_3 (NPK 8:8:6) at vegetative growth stages but non-significant difference was noted at seedling growth stage (Table 2 & Fig 5). Similarly, maximum root fresh weight (g) was recorded in T_2 (NPK 8:8:6) as compared to T_0 (control) at seedling and vegetative stages (Fig 6).

Data regarding Chlorophyll contents showed that minimum chlorophyll-a contents was present in T_2 (NPK 8:8:6) and maximum chlorophyll-a was present in T1 (NPK 4:4:3) at seedling stage (Table 3). In contrast, at vegetative stage minimum chlorophyll-a contents measured in T_0 (control) and maximum in T_1 (Fig 7). While for

chlorophyll-b, minimum contents were calculated 0.0436 mgg-1 in T_2 (NPK 8:8:6) and maximum 0.0815 mgg-1 in T_1 (NPK 4:4:3) at seedling stage (Fig 8). At vegetative growth stage (Table 4) minimum chlorophyll-b contents were calculated in T_0 (control) and maximum in T_1 (NPK 4:4:3). Minimum carotenoides contents was present in T_1 (NPK 4:4:3) and maximum carotene was found in T_0 (control) and T_1 (NPK 8:8:6) at seedling stage (Fig 9). In case of vegetative stage, maximum carotenoides was measured in T_1 (NPK 4:4:3) as compared to T_0 (control) and T_2 (NPK 8:8:6).

Chemical analysis was conducted for potassium and nitrogen. Maximum K^+ concentration (mg/g) in leaves of pearl millet was recorded in T_2 (NPK 8:8:6) as compared to T_1 (NPK 4:4:3) and T0 (control) in seedling and vegetative stages (Table 5 & 6). Minimum K^+ concentration (mg/g) in leaf was found in T_0 (control) (Fig 10). In case of stem, minimum K^+ concentration (mg/g) in stem was found in T_0 (control) and maximum K^+ concentration (mg/g) in stem was observed in T_2 (NPK 8:8:6) at both seedling and vegetative stages shown in Fig 11. Similarly in roots, maximum K^+ concentration (mg/g) in root was observed in T_2 (NPK 8:8:6) and minimum found in T_0 (control) at seedling stage (Fig 12). While at vegetative stage, maximum K^+ concentration (mg/g) in root was observed in T_1 (NPK 4:4:3) and minimum found in T^2 (NPK 8:8:6).

Data given in Figs 13, 14 and 15 showed that maximum N concentration (mg/g) in leaf was observed in T_2 (NPK 8:8:6) and minimum found in T0 (control) at seedling stage. Fig 13 showed that at vegetative stage, maximum N concentration (mg/g) in leaf was observed in T_1 (NPK 4:4:3) and minimum found in T_0 (control). In stem, Maximum N concentration (mg/g) in stem was observed in T_2 (NPK 8:8:6) and minimum found in T_0 (control) at seedling stage (Fig 14). At vegetative stage, maximum N concentration (mg/g) in stem was observed in T_2 (NPK 8:8:6) and minimum found in T_0 (control). For roots of pearl millet, maximum N concentration (mg/g) in root was 13.8 observed in T_1 (NPK 4:4:3) and minimum was 9.8 found in T_0 (control) at seedling stage. Fig 15 showed that at vegetative stage, maximum N concentration (mg/g) in root was 14.8 observed in T_2 (NPK 8:8:6) and minimum was 10.5 found in T_0 (control).

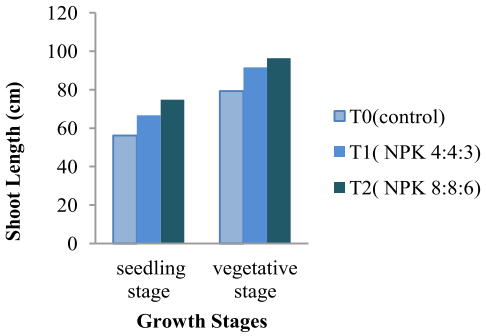

Fig 1: Effect of foliar NPK on shoot length (cm) of pearl millet

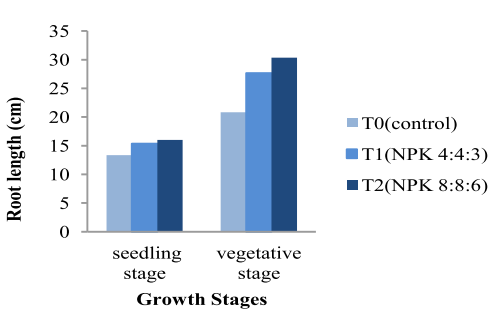

Fig 2: Effect of foliar NPK on root length (cm) of pearl millet

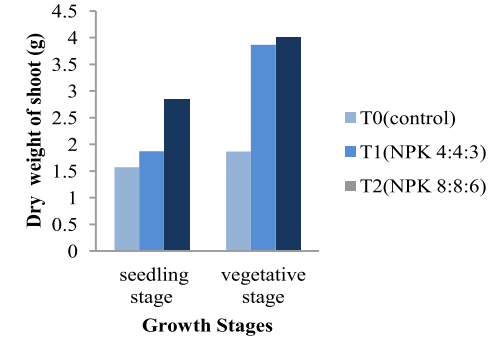

Fig 3: Effect of foliar NPK on dry weight (g) of shoot of pearl millet

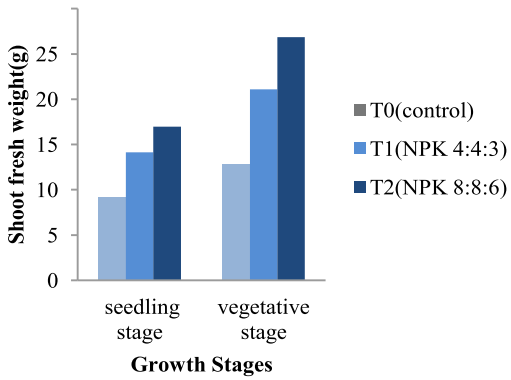

Fig 4: Effect of foliar NPK on shoot fresh weight (g) of pearl millet

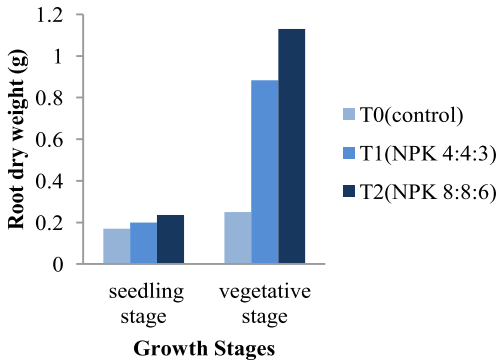

Fig 5: Effect of foliar NPK on root dry weight (g) of pearl millet

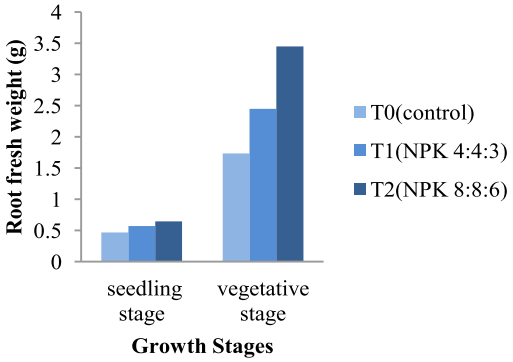

Fig 6: Effect of foliar NPK on root fresh weight (g) of pearl millet

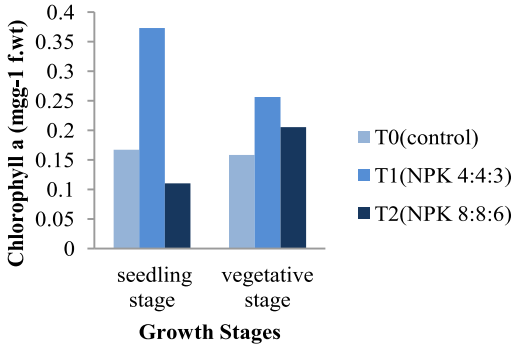

Fig 7: Effect of foliar NPK on chlorophyll a (mgg-1 f.wt) of pearl millet

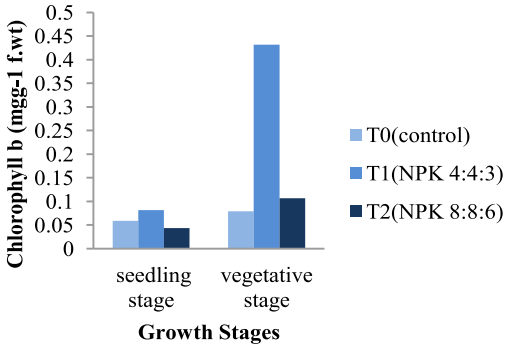

Fig 8: Effect of foliar NPK on chlorophyll b (mgg-1 f.wt) of pearl millet

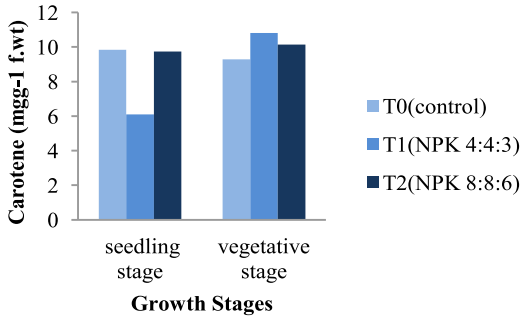

Fig 9: Effect of foliar NPK on caroteniodes (mgg-1 f.wt) of pearl millet

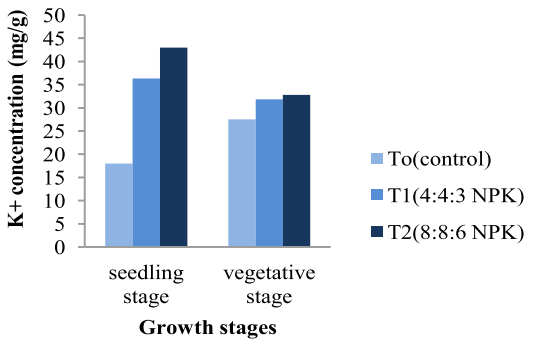

Fig 10: Effect of foliar NPK on K+ concentration (mg/g) in leaf of Pearl millet

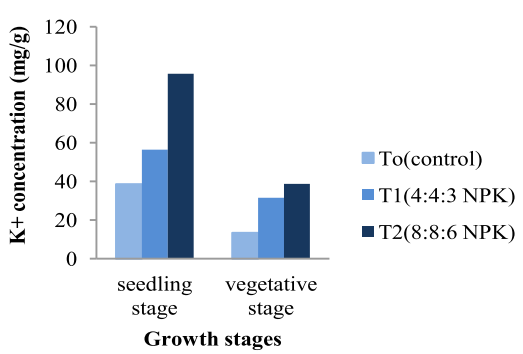

Fig 11: Effect of foliar NPK on K+ concentration (mg/g) in stem of Pearl millet

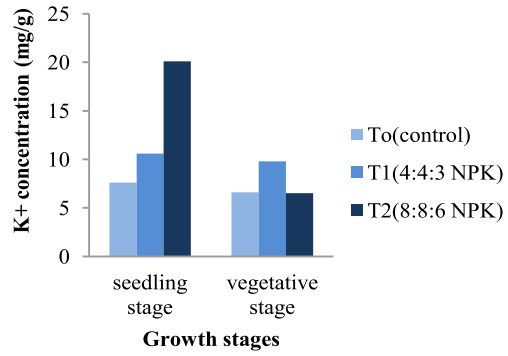

Fig 12: Effect of foliar NPK on K+ concentration (mg/g) in root of Pearl millet

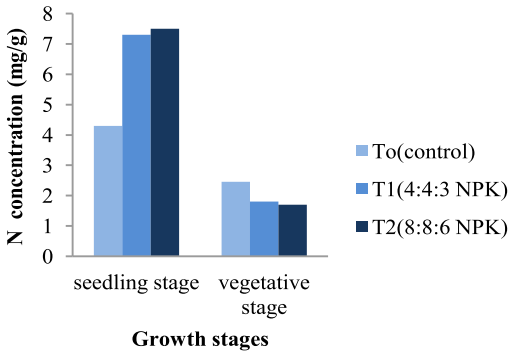

Fig 13: Effect of foliar NPK on N concentration (mg/g) in leaf of Pearl millet

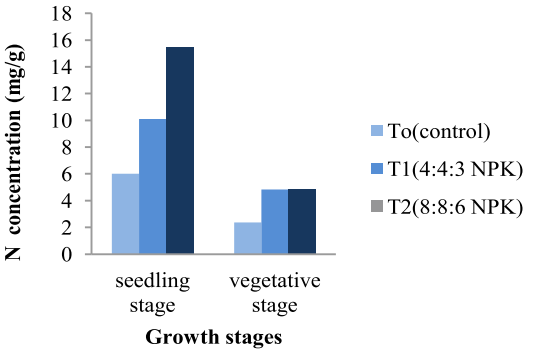

Fig 14: Effect of foliar NPK on N concentration (mg/g) in stem of Pearl millet

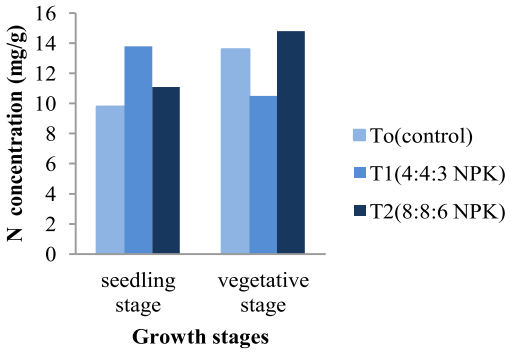

Fig 15: Effect of foliar NPK on N concentration (mg/g) in root of Pearl millet

Table 1: Means squares (MS) from the Analysis of Variance (ANOVA) for growth attributes at seedling stage of Pearl millet (*Pennisetum glaucum* L. (R.Br) under the applications of NPK

Source	df	MS of shoot length	MS of root length	MS of shoot fresh weight	MS of shoot dry weight	MS of root fresh weight	MS of root dry weight
Main Effects NPK	2	216.75**	7.124^{ns}	125.220**	2.456**	6.589***	11.269**
Error	15	21.25	2.145	16.452	3.457	0.896	1.238
Total	17						

Table 2: Means squares (MS) from the Analysis of Variance (ANOVA) for growth attributes at vegetative stage of Pearl millet (*Pennisetum glaucum* L. (R.Br) under the applications of NPK

Source	df	MS of shoot length	MS of root length	MS of shoot fresh weight	MS of shoot dry weight	MS of root fresh weight	MS of root dry weight
Main Effects NPK	2	312.478***	8.498**	246.34**	3.164ns	8.154**	13.146**
Error	15	42.569	4.516	25.649	4.265	1.245	2.1458
Total	17						

Table 3: Means squares (MS) from the Analysis of Variance (ANOVA) for pigments at seedling stage of Pearl millet (*Pennisetum glaucum* L. (R.Br) under the applications of NPK

Source	df	MS of Chl. a	MS of Chl. b	MS of total chl.	Ms of carotenoids
Main Effects NPK	2	0.014**	1.120**	7.145**	21.523*
Error	15	0.002	0.279	3.987	8.997
Total	17				

Table 4: Means squares (MS) from the Analysis of Variance (ANOVA) for pigments at vegetative stage of Pearl millet (*Pennisetum glaucum* L. (R.Br) under the applications of NPK

Source	df	MS of Chl. a	MS of Chl. b	MS of total chl.	Ms of carotenoids
Main Effects NPK	2	0.0345***	2.133*	8.799**	38.449*
Error	15	0.045	0.336	5.622	8.765
Total	17				

Table 5: Means squares (MS) from the Analysis of Variance (ANOVA) for ion concentrations at seedling stage of Pearl millet (*Pennisetum glaucum* L. (R.Br) under the applications of NPK

Source	df	K^+ conc in leaf	K^+ conc in stem	K^+ conc in root	N conc in leaf	N conc in stem	N conc in root
Main Effects NPK	2	4.877**	0.998**	3.422*	1.488*	6.221**	1.879*
Error	15	0.045	0.456	0.411	0.895	0.897	0.144
Total	17						

Table 6: Means squares (MS) from the Analysis of Variance (ANOVA) for ion concentrations at vegetative stage of Pearl millet (*Pennisetum glaucum* L. (R.Br)]under the applications of NPK

Source	df	K^+ conc in leaf	K^+ conc in stem	K^+ conc in root	N conc in leaf	N conc in stem	N conc in root
Main Effects NPK	2	6.556***	2.638*	4.289**	2.459*	7.116**	2.112**
Error	15	0.885	0.826	0.127	1.254	1.249	0.597
Total	17						

DISCUSSION

Foliar applications of NPK had significant effect on the morphological growth parameters of Pearl millet at both seedling and vegetative stages. It was noted that shoot length, root length, fresh and dry weight of shoot and root significantly increased by foliar spray of NPK at both growth stages. Javaid et al., (2006) also reported similar results that NPK application increased the shoot and root in black gram and Ojeniyi et al., (2009) reported same results in Cassava.

Increase in the growth parameters by the applications of NPK might be due to the nitrogen which played a crucial role in the construction of amino acid compounds and proteins (Miller and Donahue, 1990; Salisbury and Ross, 1992). Potassium has its major role in the photosynthetic e^- transport chain (Suksri, 1998). The effect of phosphorus and potassium on physiology and nutrition development in spinach was studied by Cengiz et al., (2001). Islam et al., (2003) used 0.1% KNO_3 as foliar spray on jute plant leaves and obtained good results in relation to growth.

The high contents of chlorophyll A and B were detected in Coronaiki fertilized with NPK foliar spray treatments which showed a relative highest value of both chlorophyll A and B contents as compared to water spray (control) treatment (Osman, 2010). Aly (2005) found that all treatments of soil nutrients N, P, K and Mg increased the leaf chlorophyll-a and "carotene" contents. On the other hand Osman, (2010) found that lowest increase in leaves carotene content over the control was exhibited by Manzanillo olive trees fertilized with NPK foliar spray. With the foliar applications of NPK, concentrations of N and K^+ increased in pearl millet. It was sound documented from previous studies that the applications of N and K improved the capability of plants to avoid or resist to uptake of N (Khan and Ashraf, 1988; Ashraf et al., 1999; 2002). Ashraf et al., (2008) also reported that the applications of N fertilizers significantly enhanced K^+ contents in sugarcane at levels of N (100 and 200 kg N ha^{-1}) under normal conditions. However, maximum K^+ was noted at the highest levels (200 kg N ha^{-1}).

CONCLUSION

From this study, it was concluded that foliar applications of NPK were useful for the enhancement of growth attributes. High concentrations of NPK (8:8:6) yielded

good results as compared to low concentrations of NPK (4:4:3).

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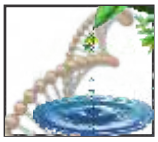

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Response of Microbes Associated with Internal and External Surfaces of *Blattella germanica* towards Hydrophobicity and Autoaggregation

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ABSTRACT: *German cockroaches (Blattella germanica) are distributed worldwide. Pathogens present on cockroach have always raised safety concerns. Present study aimed to check the bacterial autoaggregation and hydrophobicity potential. Cockroaches were trapped from house sewerage of Lahore, Pakistan in March 2016 and bacteria were isolated from external as well as internal part of cockroach. For isolation from internal colon part of digestive tract were serially diluted and selected (IM1 and IM3) from bacterial growth on agar plates while isolates from external surfaces of cockroach were selected by surface swabbing (EM4, EM5 and EM6). Morphological and biochemical characterization of isolates showed diverse morphology. The auto aggregation was recorded in all isolates after 4 hours of culture incubation. Results of hydrophobicity assay showed that isolates IM1, IM3, EM5 showed 98.09 %, 80.5 %, 90.33 % affinity towards toluene, respectively. Bacteria commonly associated with common pests, cockroaches showed the tendency to adhere to different biotic and abiotic surfaces. Isolate IM3 showed 35 % auto-aggregation after 4 hours while isolate EM4 showed 47 % aggregation as compared to rest of isolates. Result showed that on the basis of bacterial cell surface hydrophobicity and auto aggregation tendency of cells towards adherence and aggregation on host increases.*

Key Words: *Blattella germanica, Hydrophobicity, Xylene, Toluene, auto aggregation*

INTRODUCTION

Among the different household pests, the German cockroach (*Blattella germanica*) are considered as significant pests (Whitehouse et al., 2002). Infestations related to German cockroaches have been reported to be related to allergic respiratory morbidity (Sohn and Kim, 2012). Among the ubiquitous species of cockroaches, the *German cockroach* (*Blattella germanica* L.) is the most dominant specie present in the houses, hospitals and food centers. Due to their omnipresent nature, number of bacterial pathogens has been found to be reported to be associated with these cockroach species (Moges et al., 2016). Prevalence of this cockroach species act as vector in disease spreading (Solomon et al., 2016). *German cockroach* (*Blattella germanica*) are reported as important public health related insect pests (Zhou et al., 2014).

The generally reported phenomena observed is microbial aggregation that results in clustering of microorganisms of same species. Another method where bacteria can adhere to different surfaces is their ability to show hydrophobicity. Microorganisms have strategies to show hydrophobic effect to adhere to different surfaces (Doyle, 2000) and this is also the main force that involved in adhesion of different disease causing bacteria (Kouidhi et al., 2010).

Bacterial aggregation is a common mechanism observed in bacteria for the survival and adhesion (Kos et al., 2003). Probiotic bacteria have been reported to show high auto aggregation that is helpful for surface colonization as reported previously by Janković et al;(2012) where auto aggregation ability of probiotic bacteria helps in bacterial adhesion to intestinal cells. The objective of the study was to evaluate the comparative efficacy of different microbial isolates associated with cockroaches. The design of the study was to identify bacterial flora from German cockroaches and their

hydrophobicity and aggregation potential to study their colonization potential.

MATERIALS AND METHODS

Bacterial Sampling, Isolation and Characterization

Cockroaches (*Blattella germanica*) were trapped from house sewerage of Lahore, Pakistan in the month of March, 2016 and transported to laboratory for the isolation of bacteria.

Bacterial Isolation from External Surface of Cockroach

Bacteria from external surface of the cockroach were isolated with the help of sterile swab and spreaded on LB agar media (Gerhardt et al., 1994) in sterile conditions and plates were incubated at 37°C for 24 hours for bacterial growth. Isolated colonies were purified by quadrant streaking.

Bacterial Isolation from Internal Surface of Cockroach

The cockroaches were dissected using sterile dissecting tools. The digestive system of cockroach was exposed. One gram of the dissected part from the colon portion of the digestive track was serially diluted and 100 µl suspensions from 107 dilutions were used for spreading on LB agar media (Gerhardt et al., 1994) separately. Isolates were obtained from plates incubated at 37^{o}C (24 hours). Selected colonies from internal and external surface of cockroaches were purified by quadrant streaking method.

Morphological and Biochemical Characterization of Bacterial Isolates

Cultural characteristics for both of these selected isolates were done as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Biochemical characterization of the bacterial isolates were done by starch hydrolysis test, Citrate utilization test, Litmus milk reaction, Methyl Red Test, Voges, Proskauer test, Catalase test (Tittsler and Sandholzer, 1936).

Hydrophobicity Assay

This assay was performed following the method of Abdulla et al., 2014. Sterilized L-broth (10 ml) was inoculated using 100 µl from fresh culture broth (OD 0.5 at 600 nm) and incubated for 24 hours in shaking incubator. Cells were harvested (centrifugation for 20 minutes at 2000 rpm) from bacterial culture broth. The pellet was washed using sterile phosphate buffer saline twice. Finally the cells were resuspended in buffer to make final cell density of 1.0 at 540 nm. To one ml of bacterial suspension 1 ml of each of hydrocarbon (Xylene, Toluene) was added. This step was followed by rigorous shaking for 30 seconds as previously described. The test tubes were allowed to stand for 30 minutes. Optical density (OD 540 nm) of the aqueous phase was recorded after phase separation. Hydrophobicity was calculated by using following formula;

% Hydrophobicity = (A540 initial – A540 aqueous phase)/A540 initial] × 100

Auto Aggregation Assay

This assay was perfomed by following the method of Abdulla et al., 2014. Isolates were cultured in LB-Broth at 37°C for 24 hours in shaking incubator. Cells were harvested from the cultures (centrifugation at 2000 rpm for 20 minutes) and washed with phosphate buffer saline twice and suspended in phosphate buffered saline. Autoaggregation was determined during 5 hours of incubation at room temperature. After different time intervals of 1, 2, 3 and 4 hours, 100 µl from upper suspension was taken and transferred to tube with phosphate buffer saline and absorbance was measured at 600 nm. Autoaggregation was calculated by using following formula;

$\%\text{Autoaggregation} = 1 - (At / A0) \times 100$

Statistical Analysis

In all the experiments the data was analyzed statistically (Steel and Torrie, 1981). by calculating the mean values of replicates and measuring the standard errors of the means. The error bars are shown in each figure.

RESULTS AND DISCUSSION

Bacterial Morphological and Biochemical Characterization

Morphological characteristics of five isolate from external and internal surfaces of cockroach were recorded. All isolates showed circular form and entire margins as colony characteristics (Table.1). When colonies were analyzed for colony pigmentation, off white (IM1), yellow (IM3), Orange (EM4) and white (EM6) colonies were observed. Isolates either showed flat (IM1 and EM5), Convex (IM3, EM6) or raised (EM4) elevation (Table 1). Results of simple staining showed that all isolates were cocci (Table 2). The gram staining behavior showed that IM1, and EM5 were gram negative while IM3, EM4 and EM6 were gram positive bacteria. All bacterial isolates were non-spore farming and non-capsulated. Results of starch hydrolysis tests showed that all isolates IM1, IM3, EM4 and EM5 showed negative results while isolate EM6 showed positive results as visible in the form of clear zones. Citrate Utilization results showed positive response for IM1, IM3 and EM5 while negative results were obtained for isolates EM4 and EM6. The results of Litmus Milk Reaction for isolates were positive for all isolates. Results of Methyl Red Test showed that isolates, EM4, EM5 and EM6 were positive while IM1 and IM3 showed negative results. EM4, EM5 and EM6 were VP positive while IM1 and IM3 were Voges-Proskauer test negative. All the isolates were catalase positive (Table 3). Morphological characteristics like shape, flagellar arrangement and staining are the important parameters for bacterial identification (Tshikhudo et al., 2013). Cockroaches carry many pathogenic and non-pathogenic bacteria. Many species of bacteria have been isolated from cockroaches *such as Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia and Streptococcus* spp. Cockroaches act as mechanical vector in transmitting *Salmonella, Cryptosporidium parvum* and *Shigella* bacteria that cause diarrheal diseases (Salehzadeh et al., 2007). Diverse morphology and biochemical characterization was observed in these associated microbes present on cockroach surface (Menasria et al., 2014)

Table 1 : Culture characteristics of bacterial isolates from internal and external surface of Cockroaches

Strains	Form	Pigmentation	Margin	Elevation
IM_1	Circular	Off white	Entire	Flat
IM_3	Circular	Yellow	Entire	Convex
EM_4	Circular	Orange	Entire	Raised
EM_5	Circular	White	Entire	Flat
EM_6	Circular	White	Entire	Convex

Table 2: Morphological characterizations of bacterial isolates from internal and external surface of cockroaches

Bacterial isolates	Simple Staining	Gram staining	Spore staining	Capsule staining
IM_1	Coccus	Gram-ve	Non-spore forming	No capsule
IM_3	Coccus	Gram +ve	Non-spore forming	No Capsule
EM_4	Coccus	Gram+ve	Non-spore forming	No capsule
EM_5	Coccus	Gram-ve	Non-spore forming	No capsule
EM_6	Coccus	Gram+ve	Non-Spore forming	No capsule

Table 3: Biochemical characterizations of bacterial isolates from internal and external surface of cockroaches

Strains	Starch Hydrolysis	Citrate Utilization test	Litmus milk reaction	Methyl red test	Voges-Proskauer test	Catalase test
IM_1	-ve	+ve	Alkaline	-ve	-ve	+ve
IM_3	-ve	+ve	Alkaline	-ve	-ve	+ve
EM_4	-ve	-ve	Alkaline	+ve	+ve	+ve
EM_5	-ve	+ve	Alkaline	+ve	+ve	+ve
EM_6	+ve	-ve	Alkaline	+ve	+ve	+ve

Hydrophobicity Assay

Isolate IM1 showed highest percentages with xylene (98.09 %) and toluene (98.09 %).

Isolate IM3 (80.5%), EM5 (90.3%) with xylene and EM6 showed maximum hydrophobicity (84%) with toluene (Fig. 1). Hydrophobicity assay was performed to determine whether strains are hydrophilic or hydrophobic. Their affinity towards organic solvent (xylene and toluene) was also tested. Isolates response towards xylene showed that IM1,

EM5 and EM6 were strongly hydrophobic. IM3 and EM4 were hydrophilic towards xylene. IM1, IM3, EM5 showed 98.09 %, 80.5 %, 90.33% affinity towards toluene respectively. Satisfactory level of hydrophobic/hydrophilic potential in microorganisms offer them advantage of degradation of hydrocarbons or biodegradable polyesters and during milk fermentation (Obuekwe et al., 2009). Hydrophobic surfaces play not only a role in the development of biofilm but also in degradation of contaminants from soil and water. In the prevention of microbial community adhesion associated with cockroach towards different surfaces, this study is an effort of a preventive approach. Because hydrophilic nature of the cells allow the cells to move towards the surfaces for their settlement however, their quantity can be controlled using disinfectants. This may be helpful for disease prevention and control associated with microbes. Bacterial cell surface hydrophobicity facilitates hydrophobic-hydrophobic interactions with the substrates (Heipieper et al., 2010). Cell surface hydrophobicity helps the cells to manage microbial adhesion by determinig hydrophobicity of bacterial cells (Krasowska and Sigler, 2014).

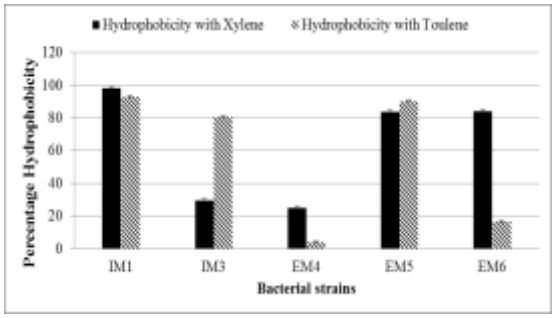

Fig 1: Percentage hydrophobicity of five isolates with xylene and toluene.

Auto Aggregation Assay

Percentages of auto aggregation was recorded after every hour of incubation and the results showed (Fig 2) that auto aggregation started increasing after first hour however, the maximum increase was recorded after 4 hours of culture incubation in all bacterial isolates except for isolate EM4 where maximum auto aggregation was recorded after 3 hours. Auto aggregation and bacterial adhesion is related to associated surface proteins. That can increase the cell hydrophobicity however; polysaccharides molecules impart hydrophilic attributes (Ortiz et al., 2015). Bacterial auto-aggregation has been reported to be important interaction involved in microbial aggregation in natural environment (Bourgeau and McBride, 1976) as well as adhesion (Pan et al., 2017). Adhesion and bacterial auto-aggregation results in aggregate formation with genetically identical cells (Grady et al., 1999)

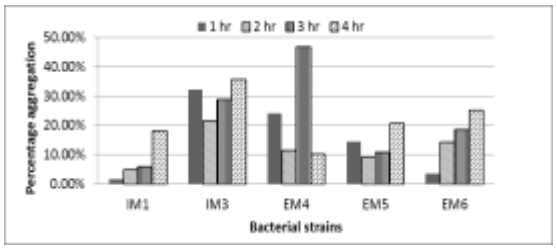

Fig 2: Percentage autoaggregation observed in five isolates after different hours of incubation

CONCLUSION

Bacteria commonly associated with common pests, cockroaches can be carriers of different diseases or food pathogenicity. The hydrophobicity percentages (>30) of these bacterial isolates showed the tendency of these isolates to adhere to different biotic and abiotic surfaces. Results showed that bacterial hydrophobicity is linked to the adherence on hydrophobic surfaces as well as adherence to host and results in bacterial aggregation on cells. Determining the microbial cell surface hydrophobicity is a fruitful approach towards cell adherence and aggregation.

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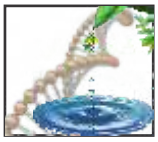

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Evaluation of Phytochemicals and Antimicrobial Potential of Clove (*Syzygium aromaticum*)

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ABSTRACT: *The flower bud of Syzygium aromaticum (clove) is one of the most imperative remedies used in indigenous medicine in Pakistan. Syzygium aromaticum is an effective drug for different ailments, and it is used as anti-gas activity, antioxidant activity, anticoagulant activity, anesthetic activity and mucus clearing activity. Antimicrobial activity of aqueous, methanol, acetone and n-hexane extracts of Syzygium aromaticum against four bacterial (E. coli, B. subtilus, S. aureus and P. multocida strains) and three fungal species (Aspergillus niger, Aspergillus flavus and Alternaria alternate) were investigated by disc diffusion method. Phytochemical analysis of Syzygium aromaticum revealed the presence of alkaloids, glycosides, flavonoids, tannic acid and cardiac glycosides. The present investigation revealed that methanol extracts had more potent activity (25 mm DIZ) than other extracts. A strong correlation was observed between phytochemicals and tested biological activities. The results of this investigation advocate that clove seed can be explored as a viable source of bioactives for the improvement of chemotherapeutic medicine against cancer in addition to acting as nutraceutical and functional food ingredient.*

Key words: Syzygium aromaticum, Clove, Antimicrobial activity, Phytochemicals

INTRODUCTION

Nature has blessed us with a wide range of economically and medicinally important flora providing food and phytomedicines (Biglari et al., 2008). Various medicinal plants have great value because of their potential utilization in folk medicine and functional foods (Siahsar et al., 2011; Hamini et al., 2014). The vaunted physiological properties of medicinal plants have been attributed to the presence of bioactive compounds such as flavonoids, phenols and tannins in different parts of the plant (Chagas et al., 2015).

Clove (*Syzygium aromaticum*) are the aromatic dried flower buds belonging to family Myrtaceae, is an evergreen plant which grows to a height ranging from 8-12 m, large oval leaves and crimson flowers in numerous groups of terminal clusters. Phytochemical analysis of the crude extract exposed the presence of alkaloids, steroids, glycoside, carbohydrates, terpenoids, resins, tannins, flavonoids and phenolic compounds (Metwally, 2014). Cloves have been used as a valuable spice in almost the entire world's cuisines. More extensively these have been utilized as folk medicine over the centuries to treat indigestion, asthma, cough, atherosclerotic, skin disorders, tooth infections and gum disease, acne, headache, wounds, insect bites, scabies and male sexual disorders (Paoli et al., 2007; Baietto 2014). The most frequently reported biological activities such as antimicrobial (Hoque et al., 2008; Javed et al., 2012), antiulcer (Metwally, 2014), anti-inflammatory and antipyretic of clove might be ascribed to the biological active constituents (Taher et al., 2015).

Recently, an extensive investigation is being focussed on extraction and isolation of phytochemicals for the development of anticancer agents, chemo preventive drugs and other nutraceuticals to fortify and supplement the physiological defence mechanisms of human body. The yield of phytoconstituents and their in vivo or in

vitro biological activities are effected by the choice of extraction solvent (Sultana et al., 2014). Thus, it would be important to study antimicrobial potential as well as phytoconstituents of clove seeds as a function of different extraction media so as to explore their potential functional food and therapeutic uses on scientific basis.

MATERIALS AND METHODS

Collection of Samples

The seeds of clove (S. aromaticum L.) were purchased from the local market of Faisalabad, Pakistan. Mature and healthy clove seeds were screened, air-dried, and pulverised into fine powder using a commercial grinder. Then the material was passed through 100-mesh sieve and was used for extraction purposes.

Extraction of Bioactives

20 grams of finely ground clove seeds were separately extracted by shaking in an orbital shaker (Gallenkamp, UK) with 100 mL of each of the four extraction solvents (100% water, 100% ethanol, 100% acetone and 100% n-hexane) for 24 hours at room temperature. The extracts were fitered and residues recovered after filtration were reextracted twice with the fresh solvents. The combined extracts were freed from solvent at 45°C in a vacuum rotary evaporator (Rotary Evaporator N-1001, EYELA,Tokyo, Japan). The solvent-free, crude concentrated extracts (CCE) were weighed to calculate the percentage yield and stored at -4ºC, for further analysis.

Qualitative analysis of Phytochemicals

The detection and extraction of different phytochemicals (Alkaloids, Flavonoids, Glycosides, cardiac glycosides and Tannic acids) of clove was also done. Alkaloids were detected by Dragondroff's reagents (Harbone, 1973) and extracted by chloroform solution. Flavonoids were detected by Siddique and Ali (1997) method whereas extracted by the method developed by Brain and Turner (1975). The glycosides and cardiac glycosides were identified and extracted by Stat-Otto procedure (Brain and Turner 1975). Tannic acids were detected and extracted by stat- Otto method.

Preparation of inoculums

The clove seed extracts were tested against a panel of microorganisms including four bacteria, *Staphylococcus aureus, Escherichia coli, Bacillus subtilus, Pasteurilla Multocida* and three pathogenic fungi, Aspergillus niger, Aspergillus flavus and Alternaria alternata obtained from Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan and identified from Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. Bacterial strains were cultured overnight at 37°C in nutrient agar (Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 30°C using potato dextrose agar (Oxoid). Spore suspensions were prepared one day before use and preserved at 4oC.

Disc diffusion assay

The antimicrobial activity of the clove seed extracts was determined by disc diffusion method (CLSI, 2012) with slight modifications. The sterilized discs (6 mm in diameter) were impregnated with 50µl extract and placed on the inoculated agar. To compare the activity with standard antibiotics, Rifampicin (30 µg/disc) (Oxoid) and Fluconazol (30 µg/disc) (Oxoid) were used as positive reference for bacteria and fungi, respectively. Disc without samples were used as a negative control. Test discs and standard disc were placed in separate petri dishes and were incubated at 37°C for 24 h for bacterial and 25°C for 3 days for fungal growth. Antimicrobial activity was evaluated by measuring the diameter of inhibition zone (mm) by zone reader.

Statistical analysis

All the experiments were carried out in thrice and the data of the investigated parameters were reported as mean ($n = 3 \times 3$) ± SD ($n = 3 \times 3$). Analysis of variance (ANOVA) was performed using Minitab 2000 Version 13.2 statistical software (Minitab Inc., PA. USA).

RESULTS AND DISCUSSION

The phytochemical analysis of clove was carried out. Results revealed (Table 1) that among all the phytochemicals, alkaloids were present in highest amount (15%) followed by glycosides (13%)> flavonoids (12%) tannic acid (11.5%)> cardiac glycosides (11%).

The present investigation (Table 2) gives an account on the antagonistic activities of *Syzygium aromaticum* water, methanol, acetone and n-hexane extract against four species of bacteria (*E. coli, B. subtilus, S. aureus and P. multocida* strains). The antimicrobial test results of the *Syzygium aromaticum* samples are shown in Table 1. The CSE were applied on bacterial strains and the diameter of inhibition zone (mm) was measured by zone reader (Table 5). From the data, it was accomplished that aqueous extracts of *Syzygium aromaticum*, was found to have an inhibitory effect against S. aureus and E. coli (22 mm and 18 mm, respectively). The methanol extract of *Syzygium aromaticum*, was effective against *E. coli* (25 mm). The acetone extract of *Syzygium aromaticum* showed highest activity against *P. multosida* (22 mm), however, n-hexane extract (150 mg/mL) of clove seeds showed strong activity against *E. coli* (17 mm)

The antifungal potential of clove seed extracts (CSE) in different solvent systems was also evaluated against *Aspergillus niger*, *Aspergillus flavus* and *Alternaria alternate*. Results of the present study (Table 3) revealed that water CSE (150 mg/mL) showed strongest activity against *A. flavus* (23 mm DIZ). Whereas methanolic and acetone CSE showed strong activity against *A. alternate* (23mm and 22mm, respectively). n-Hexane extract (150 mg/mL) of clove seeds showed strong activity against *A. niger* (20 mm DIZ). This tends to express that the active ingredients of the *Syzygium aromaticum* may be better extracted with methanol then other solvent. Test organisms were found to be more sensitive towards *Syzygium aromaticum*.

The antimicrobial potential of phytochemicals was also evaluated against four bacterial strains (*E. coli, B. subtilus, S. aureus* and *P.multocida* strains) and three fungal strains (*Aspergillus niger, Aspergillus flavus* and *Alternaria alternate*). Results showed (Table 4) that alkaloids and glycosides showed highest antibacterial activity against *B. subtilus* with 30mm and 28mm DIZ. Whereas flavonoids and tannic acids showed potential activity against *P. multosida*. However, all phytochemicals showed less antibacterial activity against *S. aureus*.

While comparing the results (Table 5) of phytochemicals against fungal strains, alkaloids and tannic acid showed strong antifungal activity against *A. alternate* with 24mm and 21mm DIZ respectively. However, flavonoids and glycosides showed strong activity against *A. flavus* with 18mm and 19mm DIZ. Results of the present findings are also in agreement with the previous findings of Javed et al. (2012) who reported that essential oils of clove exhibited strong antimicrobial activity against *B. subtilus* and *E. coli*.

The antimicrobial potential of Syzygium aromaticum is might be due to tannins, saponins, phenolic compounds and flavonoids. Most of the biological activities of plants such as antimicrobial, antioxidant, anti-aging, anticarcinogenic, antiapoptosis, anti-inflammatory, anti-artherosclerosis, cardiovascular protection, improvement of the endothelial function as well as inhibition of cell proliferation and angiogenesis can be attributed to their intrinsic reducing abilities (Karuppiah and Rajaram, 2012).

Table 1: Phytochemical analysis of clove

Phytochemicals	Qualitative analysis	Quantitative yield (%)
Alkaloids	++	15.0 ±0.23
Flavonoids	++	12.0±0.41
Glycosides	++	13.0±0.34
Tannic acid	+	11.5±0.15
Cardiac glycosides	+	11.0±0.52

Values are Mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$.

Table 2: Inhibition zones (mm) produced by various extracts of clove against different bacterial strains

Extract (mg/mL)		Bacterial strains			
		E.coli	*B. subtilus*	*P.multosida*	*S. aureus*
Water extract	50	13±0.32	10±0.31	11±0.12	16±0.33
	100	14±0.12	12±0.46	13±0.27	19±0.38
	150	18±0.21	17±0.13	15±0.43	22±0.17
Methanolic extract	50	16±0.26	14±0.68	15±0.57	14±0.42
	100	20±0.32	18±0.14	16±0.62	19±0.47
	150	25±0.38	22±0.42	19±0.39	20±0.31
Acetone extract	50	15±0.15	11±0.16	16±0.65	13±0.23
	100	18±0.25	13±0.21	19±0.16	16±0.21
	150	21±0.31	18±0.24	22±0.27	18±0.28
n-Hexane extract	50	12±0.29	9±0.17	9±0.42	11±0.37
	100	14±0.52	11±0.42	10±0.32	13±0.16
	150	17±0.17	14±0.46	13±0.10	15±0.52
Chloromphenicol		31±0.21	26±0.32	24±0.31	29±0.32

Values are Mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$.

Table 3: Inhibition zones (mm) produced by various extracts of clove against different fungal strains

Extract (mg/mL)		Fungal strains		
		A. niger	*A. flavus*	*A. alternate*
Water extract	50	11±0.31	13±0.32	11±0.16
	100	13±0.27	18±0.19	15±0.32
	150	16±0.42	23±0.28	20±0.18
Methanolic extract	50	15±0.36	15±0.31	17±0.53
	100	16±0.18	18±0.26	20±0.26
	150	18±0.21	21±0.17	23±0.43
Acetone extract	50	15±0.26	12±0.31	16±0.28
	100	16±0.22	16±0.32	18±0.16
	150	18±0.26	19±0.12	22±0.32
n-Hexane extract	50	13±0.17	9±0.51	12±0.18
	100	17±0.29	11±0.36	13±0.24
	150	20±0.42	14±0.60	18±0.19
Fluconazole		24±0.27	26±0.28	29±0.43

Values are Mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$.

Table 4: Inhibition zones (mm) produced by various Phytochemicals of clove against different bacterial strains

Phytochemicals (100mg/mL)	Bacterial strains			
	E.coli	*B. subtilus*	*P. multosida*	*S. aureus*
Alkaloids	23±0.15	30±0.29	27±0.31	25±0.24
Flavonoids	17±0.21	25±0.31	31±0.28	18±0.17
Glycosides	21±0.27	28±0.24	23±0.32	18±0.29
Tannic acid	24±0.32	23±0.17	29±0.37	23±0.41
Cardiac glycosides	27±0.28	20±0.37	25±0.53	25±0.23
Chloromphenicol	40±0.29	38±0.41	36±0.42	42±0.21

Values are Mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$.

Table 5: Inhibition zones (mm) produced by various photochemical of clove against different fungal strains

Phytochemicals (100mg/mL)	Fungal strains		
	A. niger	*A. flavus*	*A. alternate*
Alkaloids	17±0.32	22±0.17	24±0.19
Flavonoids	12±0.37	18±0.34	17±0.38
Glycosides	16±0.42	19±0.71	13±0.32
Tannic acid	19±0.27	16±0.45	21±0.21
Cardiac glycosides	18±0.15	15±0.36	16±0.17
Flucanazole	24±0.23	26±0.41	29±0.14

Values are Mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$.

CONCLUSION

In the present study phytochemical screening and antimicrobial activities of different solvent extracts from clove seeds were appraised. The clove seed phytochemicals and extracts produced by the tested four extraction media exhibited considerable antimicrobial capabilities. Specifically, methanol extracts offered the best antimicrobial potential advocating the use of clove for the development of target and specific anticancer drugs and neutraceuticals to treating health related disorders.

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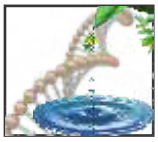

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Fecal Matter as a Bio-indicator of Heavy Metal Toxification in Punjab Urial

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ABSTRACT: *Heavy metals are a major class of pollutants that are responsible for high level of toxicity in living beings. These metals have the tendency to bio-accumulate in the living tissues; their levels can be determined in the various organs of the body. Most of the old methods used for the indication of heavy metal contamination in the environment usually involve the killing of animals, whereas current study is used to determine the heavy metal contamination in fecal matter, feed, water and soil without causing any harm to the lives of animals. Observed level of heavy metal like cadmium (0.0073 ppm to 0.020 ppm), lead (0.029 ppm to 0.036 ppm), zinc (4.88 ppm to 5.326 ppm) and copper (0.118 ppm to 0.135 ppm) showed that their amounts are significantly high in fecal sample of Punjab Urial as compared to other samples collected both from Lahore zoo as well as Bahawalpur zoo.*

Key Words*: Lahore zoo, Bahawalpur zoo, atomic absorption spectrometry*

INTRODUCTION

Environmental pollution is a major problem for the living organisms. Various chemicals such as carbon monoxide, sulfur dioxide, nitrogen oxides, volatile organic compounds, ozone, heavy metals and irrespirable particulate matter escaped to the environment. Some pollutants like heavy metals have caused a change in the distribution of certain wildlife species. The increased amount of heavy metals in the environment is due to many sources, which include atmospheric deposition, sewage irrigation, improper stacking of the industrial solid waste, mining activities, the use of pesticides and fertilizers. The vast use of pesticides which contain Hg, As, Cu, Zn and other heavy metals are also polluting the environment (Arao et al., 2010). Heavy metals are present naturally in earth crust and are persistent environmental contaminants since they cannot be destroyed and degraded. Since these elements do not decay with time their emission to the environment is a serious problem which is increasing worldwide due to the rapid growth of population, increased combustion of fossil fuels, and the expansion of industrial activities (Smodis and Bleise, 2000). The captive animals are also facing the same problems. There are many harmful effects of heavy metals when they exceed the bio-recommended limits (Duruibe, 2007; Chao et al., 2014). The exposures of captive animals to highest concentration of Pb was noticed in mammals close to urban area with heavy traffic and are also near metal mines and smelters (Gupta, 2013). Different studies have also reported various concentrations of metals in wild mammals living in highly contaminated area near different industrial areas (Pokorny and Ribaric., 2000; Roux and Marra, 2007; Beyer et al., 2007; Dzugan et al., 2012). Most of these studies involved killing of wildlife species as biological indicators to detect the presence of the toxicants in their own ecosystem. However the method of sacrificing or killing of animal may appear more scientific, but is certainly ethically unsound. Given the concern for loss of animal lives for scientific investigation, and the increasing biological poverty of the planet earth, there is an urgent need for developing biological indicator which will not involve killing of animals. To overcome this problem it was proposed to use fecal matters as bio-indicators

to study their exposure toward heavy metals (Gupta and Bakre, 2013). It is common observation that most of the zoos in Pakistan are near the main roads (Mall and GT road) and mostly surrounded by human habitations so the animals housed in zoo sharing the urban air need to be paid attention. The animals especially the mammals which lives in zoo may be affected everyday by a number of uncountable vehicles passes on these roads. Most of these vehicles are petrol driven and include buses, cars, motorcycles and scooters. The cages of animals are too close to the road. The automobile on this road continuously emitting exhaust and since the animals are restricted to cages and they continuously exposed to the automobile exhaust. With the development of economy and society, heavy metal contamination has been commonly increasing in the world. It is almost a serious threat to every country. In the world's top ten environmental events, two events have been related to heavy metal contamination (Yang and Sun, 2009). The automobiles exhaust is one of the sources of heavy metals along with toxic gases (Falahi-Ardakani, 1984).

In free living animals and in caged animals in zoos study of environmental contamination is a difficult risk. This is primarily because of difficulty in obtaining samples, which can be at the most opportunistic, from these animals. Therefore for the study of environmental contamination particularly feces of wild animals can be used as biological indicator and analysis of feed and water along with the soil in cages which is receiving particulate air pollutants can be used for indication of source that air pollution is the primary cause due to high density of traffic in the city (Gupta and Bakre, 2012). Keeping in mind the present study was designed to find the effect of heavy metals as pollutants on the survival of wild animals such as Punjab Urial which are kept in captivity and one of the endangered species of Pakistan. Main objective of present study is the analysis of fecal matter, feed and water with the soil in cages which is receiving heavy metal contamination from the air. As most of the studies are associated with the loss of animal's lives for scientific investigation of environmental contamination, there is crucial need for developing biological indicator which will not link with the killing of animals. The current study was therefore planned to detect levels of heavy metals in fecal samples so as to determine the applicability of this method over detecting metals in animal tissues.

MATERIALS AND METHODS

Study Area

The Study area selected for research was Lahore and Bahawalpur Zoo. Lahore Zoo (31.556006°N/74.325959°E) is a world's 3rd oldest zoo opened in 1872 (Nemat et al., 2015). It is located on Mall road Lahore in Punjab, Pakistan. Lahore Zoo covers an area of 98 ha, with 1400 of animals of containing 133 different species (Hussain et al., 2015). Annual visitors of Lahore Zoo are 3 million. On the other hand Bahawalpur zoo (29°4028°N/71.62827°E) opened in 1942 and also covers an area of 25 acres (10 ha) with total number of 870 animals (World Heritage Encyclopedia). Deers house were selected for research purpose in both zoos the enclosures are located near the roads. Consequently, vehicular exhaust and dust from the road can accumulate on the surface of enclosures and increase the pollution levels.

Selection of Animals

Total three Urial (*Ovis orientalis punjabiensis*) *i.e.* 1 male, 1 female and 1 fawn were selected from Bahawalpur zoo whereas nine (2 Urial male, 4 female and 3 fawns) were selected from Lahore zoo.

Sampling

The fecal matter of selected animals was collected in properly labeled polythene bags. Soil sample was also collected from the enclosure of both zoos at the depth of 6 inch, 12 inch and 18 inch. All collected samples were air dried and grinded before chemical digestion. Water from the enclosure was collected in sterilized and tightly closed bottles for further analysis.

Heavy Metals Detection

Chemical analysis of cadmium, lead, zinc and copper was done with the help of Graphite Furnace Atomic Absorption Spectroscopy at 228.9 nm, 217 nm, 213.9 nm and 324.7 nm respectively. The fecal, soil and water samples were digested prior to heavy metal analysis by following Gupta, 2013.

RESULTS

Levels of heavy metals including lead, cadmium, copper, chromium and zinc were determined in fecal matter of Urial (*Ovis orientalis punjabiensis*) kept under captivity of Lahore and Bahawalpur zoo. At Lahore zoo cadmium level was higher (0.008 ppm) in 6 inch soil sample and lowest level (0.00067 ppm) was detected in 18 inch soil sample (Fig 1). The observed concentration of cadmium in fecal matter and 12 inch soil was almost same (0.007 ppm) as shown in Fig 1. Regarding lead again highest concentration (almost 0.12 ppm) was observed in soil sample collected from the depth of 6 inch (Fig 2) in Lahore zoo whereas the lowest of lead was determined in feed. Concentration of zinc was also high (9.26 ppm) in soil collected from the depth of 6 inch like cadmium and lead (Fig 3). Lowest level (0.001 ppm) of zinc was determined both in feed and water sample (Fig 3). The observed level of zinc in 12 and 18 inch soil sample was same (0.06 ppm). Like other three heavy metals cadmium, lead and zinc level of copper was also high in soil sample collected from 6 inch i.e. between the values of 0.25 to 0.3 ppm (Fig 4). Whereas the observed level of cadmium, lead, zinc and copper was 0.0073 ppm, 0.029 ppm, 5.326 ppm and 0.135 ppm respectively in fecal sample collected from Urial of Lahore zoo (Fig 1-4). Statistical analysis showed that the observed amount of heavy metal in fecal sample was significantly related the amount observed in environmental samples i.e. soil, water and feed. In Bahawalpur zoo higher level of cadmium was detected in fecal matter where as in 18 inch soil sample it is present in small level (Fig 5). In water and 12 inch soil sample their amounts were same 0.01 ppm as shown in fig 5. In Bahawalpur zoo largest amount of lead was detected in fecal matter 0.035 ppm and in soil taking from the depth of 18 inch lead was not determined (Fig 6). Highest level of zinc was detected in 18 inch soil and lowest level of zinc was detected in water sample collected from Bahawalpur zoo (Fig 7). In soil collected at the depth of 6 inch large concentration of copper was detected near about 0.17ppm and smallest level of copper was detected in water near about 0.01 ppm at Bahawalpur zoo (Fig 8). Whereas in fecal matter level of copper was also slightly higher, its amount was 0.12 ppm and in feed sample copper amount was above 0.1 ppm (Fig 8). Significant association was observed between the tested variable as determined by one way ANOVA (Table 1 and 2).

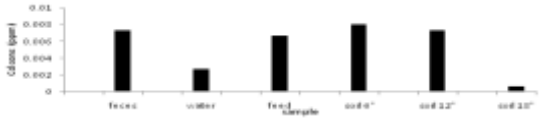

Fig 1: Cadmium concentration in different samples of Lahore Zoo

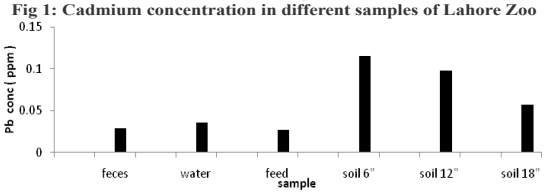

Fig 2: Lead concentration in different samples of Lahore Zoo

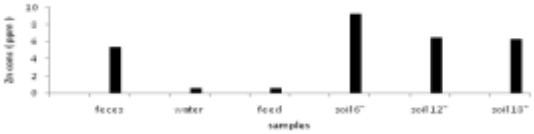

Fig 3: Zinc concentration in different samples of Lahore Zoo

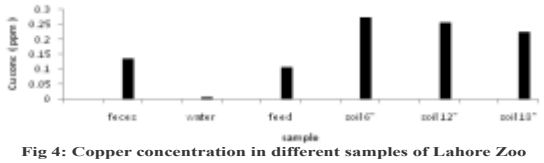

Fig 4: Copper concentration in different samples of Lahore Zoo

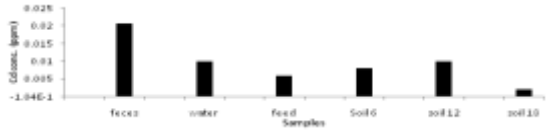

Fig 5: Cadmium concentration in different samples of Bahawalpur Zoo

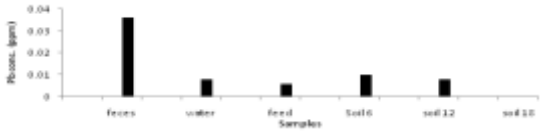

Fig 6: Lead concentration in different samples of Bahawalpur Zoo

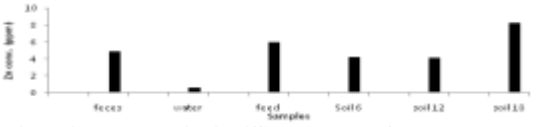

Fig 7: Zinc concentration in different samples of Bahawalpur Zoo

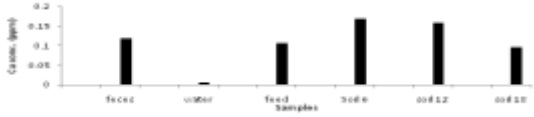

Fig 8: Copper concentration in different samples of Bahawalpur Zoo

Table 1: One way ANOVA to determine association between the measures variables of Lahore zoo

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	62.501	3	20.834	19854.295	.000
Within Groups	.008	8	.001		
Total	62.510	11			

Table 2: One way ANOVA to determine association between the measures variables of Bahawalpur zoo

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	80.865	3	26.955	4124.694	.000
Within Groups	.052	8	.007		
Total	80.917	11			

DISCUSSION

Heavy metals are an important class of pollutants present in the atmosphere. Their bioaccumulation in the living tissues makes them a potential hazard for the health of living beings. Exposure to vehicular exhaust, industrial emissions and various other sources is inevitable in the present day life and it is necessary to determine the exposure levels in a day so that proper mitigation and reduction measures may be taken. The present study therefore focused on the feasibility of using fecal matter as an indicator of heavy metal exposure on a day-to-day basis. Fecal matter is easy to collect and does not require killing or dissecting the animal. Therefore it can be used as an alternate bioindicator for heavy metal exposure in living beings (Gupta and Bakre, 2013).

In the current study varying trace metals were detected in fecal, feed, water and soil from various depths. And the samples were collected from two different locations Lahore and Bahawalpur zoo, different levels of heavy metals detected from these samples and heavy metals including cadmium, lead, zinc and copper were studied. The purpose to select these heavy metals was due to their release into the environment from different sources such as industries and particularly vehicles. The study of different samples of Bahawalpur zoo showed that the concentration of cadmium was not very high even but still fecal samples had greater amount of cadmium as compared to water, feed and soil. The higher level of Cd in feces may be due to inhalation of cadmium from the air. The level of zinc compared in all samples which were taken from the Bahawalpur and Lahore zoo was high in feces and feed in both locations. Overall level of zinc in different samples was also high as compared to other metals such as Cd, Pb and Cu. The high values of Zn showed that this metal was discharged or abundantly present in the atmosphere. The source of Zn in atmosphere may be due to vehicles tyers, that Zn is usually produced by the vehicles of tyers.

CONCLUSION

The observed amount of heavy metal in the fecal sample was considerably high in fecal sample as compared to the other samples in both zoos. The recorded values of heavy metals in fecal samples was significantly correlated with the observed values of other samples i.e. water, feed and soil sample. From the current study it was concluded that fecal matter can be used as an alternate bioindicator for heavy metal exposure in living beings.

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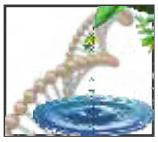

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Effective Microbes Accelerate Granulation in Wound Healing Process of Strangles Affected Horses

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ABSTRACT: *The study was carried out to explore the role of effective microbes in curing the septic wounds of Strangles disease affected horses. The submaxillary lymph nodes abscessation resulted during the course of disease and ultimately ended into formation of wounds which were subject to septic environment. Adopting conventional methods of treatment i.e. local use of antiseptic preparations and parenteral administration of antibiotics usually takes long time in healing of wounds. At times the horses do not respond the treatment which results in loss of animal life as well as economic thrashing. Use of effective microbes in animal health is an area which needed exploration. We used effective microbes (EM Technology) orally / locally to cure the septic wounds of Strangle's affected horses and also evaluated its sensitivity in vitro. We found that Bio Vet an EM Technology product as an adjuvant therapy is highly effective for the treatment of strangle wounds. It considerably reduces the healing time by quick granulation and S. equi (causative organism) was also found sensitive to Bio Vet in vitro.*

Key Words; *Strangles, Healing, EM Technology, Equines*

INTRODUCTION

Strangles is a highly contagious and serious infection of horses and other equines caused by the bacterium, *Streptococcus equi* (Bazely 1942; Evers et al., 1968; Meyer et al., 1992; Vin et al., 2016). The disease is characterized by severe inflammation of the upper respiratory tract, with extensive swelling / rupture of the lymph nodes, which produces large amounts of thick, creamy pus. If untreated the animal may die (Bone et al., 1963; Anzai et al., 1999). Horses of all ages are susceptible, though strangles is most common in animals less than 5 years of age and especially in groups of weanling foals or yearlings. Foals under 4 months of age are usually protected by colostrum-derived passive immunity (Anzai et al., 2000). S. equi is maintained in the horse population by carrier horses but does not survive for more than 6–8 weeks in the environment. Although the organism is not very robust, the infection is highly contagious. Transmission is either by direct or indirect contact of susceptible animals with a diseased horse. Direct contact includes contact with a horse that is incubating strangles or has just recovered from the infection, or with an apparently clinically unaffected long-term carrier (Libardoni et al., 2016). Indirect contact occurs when an animal comes in contact with a contaminated stable (buckets, feed, walls, doors) or pasture environment (grass, fences, but almost always the water troughs), or through flies (Blunden et al., 1994). Since this is a contagious disease therefore prompt treatment and control is essential to save the other precious horses (Meyer et al., 1992). The mortality rate is more than 8 percent. Effective microbes can play an important role in curing this deadly disease. Susceptible horses develop strangles within 3–14 days of exposure (Meyer et al., 1992). Animals show typical signs of a generalized infectious process (depression, in appetence and fever of 39°C–39.5°C). More typically of strangles, horses develop a nasal discharge (initially mucoid, rapidly thickening and purulent), a soft cough and slight but painful swelling between the mandibles, with swelling of the submxillary lymph node (Boschwitz et al., 1994). The lymph nodes become hard and very painful, and may obstruct breathing (Timoney et al., 1985; MacKintosh et al., 1988) (Figs.1&2) This is thought to be the result of partial immunity although

this may also result from infection by *S. equi* of relatively low virulence (Anzai et al., 1999). Classic strangles is a severe infection that can be fatal, usually because of a variety of complications that occur. The main and often fatal complications of strangles include dissemination of infection to unusual sites like abdominal or lung lymph nodes, inflammation of peripheral blood vessels, anaemia and inflammation of the subcutaneous tissue (Oikawa et al., 2006). The disease has also been reported communicable to humans (Piotr and Anca, 2016). Treatment of a horse in the early stages of strangles is usually effective and is not associated with untoward effects. The causative agent is highly susceptible to Penicillin G and combination of Streptomycin / Penicillin (Mukhtar et al., 1988; Bazely, 1992; Flanagan et al., 1998). Wound healing or wound repair is the body's natural process of regenerating dermal and epidermal tissue (Adriana et al., 2015). Following wound, a set of complex biochemical events takes place in a closely orchestrated cascade to repair the damage (Lindmark et al., 1996; Garg, 2000; Midwood et al., 2004). In angiogenesis, new blood vessels grow from endothelial cells (Chantsavang and Watcharangkul 2004).

Effective microorganisms are a mixed culture of fermentative, soil based, beneficial microorganisms that can be employed to many environments to improve the health and vitality of water, soil, plants and animals. (Chantsavang et al., 2002; Chantsavang and Watcharangkul, 2004). EM is the fundamental mother culture of new products developed and produced within Australia for microbial balancing in soils and water. EM is a living entity containing active anaerobic and aerobic microbes. The most prominent organisms are photosynthetic bacteria, lactic acid bacteria and yeast (Daly and Stewart, 1999; Timmerman et al., 2006). EM also comprises fermentative fungi and Actinomycetes and has also been trialed on cash crops (Huang, 2016). The uniqueness of microorganisms and their often unpredictable nature and biosynthetic capabilities had made them likely candidate for solving particularly difficult problems in the life sciences and other fields as well. (Anjum, 1999) described a considerable increase in the production of eggs of layers offered with drinking water treated with EM. EM mixed in drinking water decreased mortality up to 35% in broiler chicken. Fecal and litter examination of EM treated layer chicken indicated lower parasitic oocyte count (Chantsavang and Watcharangkul, 2004). Most of the diarrhea cases caused by *E. coli*, typhoid cases by Staphylococcus and respiratory infections due to Pasteuralla can be treated successfully with EM culture. Bio Vet. an EM culture has positive effect on the growth rate of male Sahiwal calves (Maqbool et al., 1999). EM has a prophylactic efficacy against Avian Salmonellosis and average milk production in cattle is significantly increased and mortality is decreased by feeding hay treated with EM (Allaudin et al., 2009). Administration of EM culture orally reduced the incidence of Deg Nullah disease in buffaloes (Maqbool et al., 1999).

The equine industry is a flourishing area demanding to produce and export world class horses free from diseases. A young horse recovering from strangles is required to be saved against exposure to secondary infections caused by septic sub-maxillary lymph node wounds in the least possible time to check the secondary infections and making them fit for work/export. Foregoing in view we postulate that EM can enhance wound healing process in a dynamic manner by releasing bio active metabolites and competing the septic pathogenic organisms in wounds of strangles affected horses (Fig. 3). Effective microbes can play an important role in curing this deadly disease. Our study is focused on the use of Effective Microbes Technology (EM Technology) as an adjuvant treatment for the horses suffering from strangles.

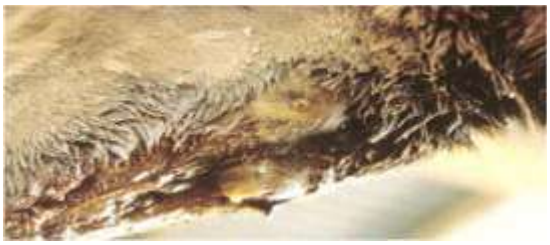

Fig 1: Ruptured and draining submaxillary lymph node

Fig. 2: A maturing abscess

Fig 3: Healing and granulating wound

MATERIALS AND METHODS

Subjects

Two to three years old thirty uncastrated, thoroughbred horses, suffering naturally from strangles with submaxillary lymph node wounds , and having body weight 280-300 kg without a lifetime history of any infectious/contagious disease are included in the study. The horses made study entry on the day of rupturing submaxillary lymph node abscesses. The horses were selected from Remount Depot Mona and Laboratory procedures were conducted at Biology Research Laboratory of Lahore Garrison University.

Materials

The EM prepration i.e Bio-Vet 20 lit was obtained from University Of Agriculture Faisalabad by the kind courtsey of Dr Azhar Maqbool Chairman Department of Parasitology, University of Veterinary and Animal Sciences, Lahore. The glass ware, autoclave, horse restraining hobbles and other lab facilities were used from Remont Depot Mona and Biology Research Laboratory of Lahore Garrison University.

Study Design

The horses were divided in two groups A and B comprising 15 animals each. Animals of group A were given available standard treatment i.e single I/M injection of Procaine Penicillin 40 lac IU mixed with 01gm Streptomycin given at the dose of 5 gm per animal for consective 7 days at 0900 hrs daily. The wounds of these animals were cleaned daily with 1% copper sulphate solution thrice a day at 1000 hrs,1800 hrs and 0200 hrs till scar formation of wound.

Animals of group B were given additional 100 ml of Bio-Vet (EM solution) orally with stomach tube and cleaning of wounds only by 40 ml of Bio-Vet Solution at the same timing as of group A.

Healing of wounds of both groups was monitored daily at 1800 hrs for scar formation by means of physical observation of growing granulation tissue.

For culture sensitivity tests nostrils discharge samples were taken aseptically from the wounds of all thirty horses of both groups by sterilized swabs during initial three days of study, which then were dipped in Nutrient broth and transported to laboratory at 25°C for sensitivity analysis and grown on culture media.The findings were recorded as very sensitive (++++) clear zone of inhibition more than 30 mm diameter, sensitive (+++) clear zone of inhibition 25-30 mm diameter, moderately sensitive (++) zone of inhibition 15-25 mm diameter with 1 or 2 colonies in the zone of inhibition, less sensitive (+) zone of inhibition 5-15 mm diameter with a few colonies of resistant microorganisims and not sensitive (-) zone of inhibition less than 5mm diameter with many colonies of resistant microorganisims were recorded.

RESULTS

Wound Healing Duration

Average healing time of wounds among group A animals was found to be 14.4 days whereas in group B it was observed to be 8.26 days The group B administered with Bio- Vet indicated decreased healing time which is the result of accelerated healing process as shown in Fig. 4.

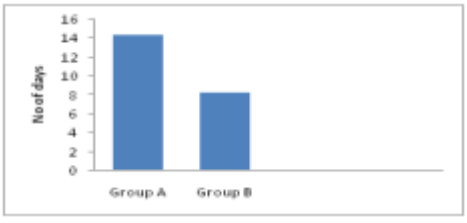

Fig 4. Effect of Bio-Vet on wound healing time.

Sensitivity Effect

S.equi was found equally sensitive to Bio-Vet compared with combination of Penicillin/Streptomycin (Combiotic) because there was no significant difference in sensitivity results, however Tetracyclin and Ampicillin were found having minimum antibacterial activity against *S.equi* as indicated in Fig. 5.

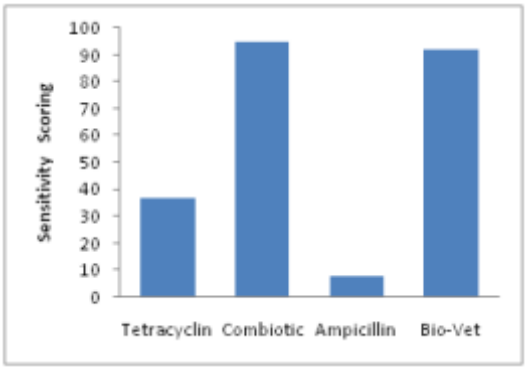

Fig 5: Sensitivity comparison of *S.equi* to Tetracycline, Combiotic, Ampicillin and Bio-Vet.

DISCUSSION

Up till now no adequate research work has been carried out for the reason that more interest for the use of beneficial microorganisms has been shown in agriculture sector. In the present study the period of healing of submaxillary lymph node wounds of strangles affected horses was reduced by treating with combiotic (Penicillin+Streptomycin) alongwith oral and local administration of Bio-Vet an EM product.

In livestock the use of Bio-Vet is sucessfully done in poultry and farm animals. Maqbool et al ., (2001) studied the effect of effective microorganisms in controlling the most important disease in cattle i.e. "Deg Nala" and experienced that the disease was very common in areas where rice straw was being used as hay for animals to feed. They concluded that in a therapeutic trial effective microoganism were given orally (2% solution) and a vasodilator (Nitroglycerine ointment) applied locally on the lesions affected the highest percentage (95%) cure rate. However in present study 100 ml Bio-Vet orally and 40 ml for the local treatment were used that gave 100% cure rate within average period of 8.26 days. Our study commensurate with these findings.

Gut flora and EM have potential to affect health and disease far beyond the gut (Takai et al., 2000). There is increasing evidence that EM have beneficial effects in preventing a wide range of conditions and improving health. We observed that in a combination with combiotic the cure rate was 100%. This study suggests that the oral administration of EM improves the gut flora and their substrates play an important role in animal health most probably through boosting the immune system or direct antibacterial activity, for which a separate study is awaited.

In the field of animal production the beneficial effects of EM on many aspects of animal production systems has been widely demonstrated in China. However, the exact mechanisms of how EM, once ingested, elicits beneficial effects on animal health, growth and metabolism is not known. To study the effect of EM without Combiotic was not found feasible keeping in view of the uncertain fate of precious horses, however the same may now be done in other animals. Improvement of microflora of the gut is also suggestive that EM can be used in preventing various infectious diseases in animals and probably in humans too.

The examination of the slides made from culture revealed Gram positive cocci and arranged in drain of various lengths. Colonies were small, smooth glistening drop like growth on nutrient agar as also observed by (Walker et al., 2002; Wizeman et al., 2001). Bio-Vet also showed excellent sensitivity against *S. equi.* Rehman et al., (2012) studied 5 different effective microorganisms against various pathogenic bacteria in-vitro and it showed good antibacterial activity against all the bacteria under study. Overall results of EM culture which are biologically active against the microorganisms suggest that skin wound infection due to *Staphylococcus* spp; and respiratory infection due to *Pasteurella* spp; can be treated with beneficial microorganisms.

The antibacterial activity of Bio-Vet against *S. equi* in early healing of strangles affected submaxillary lymph node wounds is suggestive of the sterilizing role of lactic acid produced by EM thus accelerating the granulation process and inhibiting the proliferation of pathogenic bacteria to

which the wound is exposed.

CONCLUSION

Based on the results of this study, it is concluded that Bio-Vet (EM) has a positive role in curing the strangles affected submaxillary lymph node wounds. The improvement of gut microflora resulting into beneficial metabolites and bio active substances like vitamins etc and release of lactic acid by EM are the plausible mechanisms of speedy granulation by which the wounds are healed in less than 10 days. Bio-Vet and Combiotic have synergistic activity against *S. equi.* The use of Bio-Vet in horses is quite safe and free of side effects, therefore can be used for improving their health condition. Wounds can easily be treated by oral administration of Bio-Vet with local treatment, thus reducing the expenditure incurred on antibiotics. The market price of Bio-Vet is only rupees 500 per gallon. Present study also justifies that Bio-Vet may be utilized and introduced in the country on a wide scale for the improvement of animals health.

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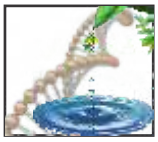

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Interaction of Sewage Bacteria with Heavy Metals, Antibiotics and Plants

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ABSTRACT: *Environment pollution of toxic heavy metals and antibiotics is widespread due of massive industrialization and overuse of antibiotics. Moreover, metal contamination can function as a selective agent in the spread of antibiotic resistance, affecting both animals and plant species. Isolation, identification and characterization of three heavy metal resistant bacterial strains from sewage pipes of Lahore city was carried out in this study. On the basis of morphology and 16S ribosomal RNA (rRNA) gene sequencing, the isolates were identified as Escherichia coli strain MHR-1, Bacillus subtilis strain MHR-2, Exiguobacterium aurantiacum strain MHR-3. These isolates were resistant to Cadmium (Cd) at (< 100 µg/mL), Chromium (Cr) (< 500 µg/mL), Lead (Pb) (< 400 µg/mL) and Arsenic (As) (< 200 µg/mL). Moreover, the isolates tolerated a combined stress of 50 µg/mL for all the heavy metals tested. Antibiotic susceptibility of the bacteria towards the 9 antibiotics revealed: MHR-1 was resistant to 66%, intermediate to 12% and susceptible to 22%; MHR-2 was resistant 66%, intermediate to 22% and susceptible to 12%; MHR-3 was resistant to 22%, intermediate to 33% and susceptible to 45%. For antibiotic-heavy metal co-stress, about 78% of antibiotics showed synergistic effects while 11% showed antagonistic effects along with a stress of 50 µg/mL of all heavy metals tested. Test of isolates for their ability to affect seed growth with and without heavy metal stress, showed no significant interaction with plants to tolerate the heavy metal stress. This study proposes an effective approach to target antibiotic resistant bacteria with combine stress of heavy metals, and further investigation will lead in to better insight in devising an effective treatment for targeting resistant pathogens.*

Key Words: *Sewage bacteria, Heavy metal resistance, 16S rRNA Sequencing, Antibiotic Susceptibility, Co-tolerance.*

INTRODUCTION:

Heavy metal pollution of natural environments is a serious environmental problem (Cheng, 2003). Pollutants from domestic and industrial wastewater can permanently damage natural ecosystems (Rehman et al., 2008). Heavy metals like Cadmium, Lead, Chromium and Arsenic are nonessential elements for living organisms and can poison plants, animals, and humans (Gupta et al., 2012). Cadmium is also one of the most toxic pollutants of the soil, released into the environment by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels (Tsai, 2006). Like Cadmium, Lead is also a major pollutant found in soil, water and air, and highly toxic to living biota (Luo et al., 2014). Similarly, hexavalent chromium Cr (VI) and trivalent chromium Cr (III) are the most widespread chromium species of the natural environment (Chiang et al., 2011). Major sources of chromium pollution include leather tanning industries, wood preservatives, chromium electroplating, alloy manufacturing, and use as corrosion inhibitor of nuclear power plants (Singh et al., 2007). As, also a toxic heavy metal element is widely distributed in nature (Nath et al, 2008). Arsenic arises from various natural sources like weathered volcanic, marine sedimentary rocks, minerals, fossil fuels, water, air, living organisms and human activities including agricultural chemicals, mining, medicinal products, wood preservatives, industry activities (Malik and Aleem, 2011). Therefore, removing such heavy metal pollutants

from waste waters is of great environmental significance.

Metal contaminants can functions as a selective agents promoting antibiotic resistance in bacteria (Baker-Austin et al., 2006). Furthermore, unlike antibiotics, metals are not subjected to degradation and can subsequently create long-term selection pressure to promote antibiotic resistance in bacteria (El-Baz et al., 2015). Thus, there are concerns that metal contamination can potentially maintain a pool of antibiotic-resistance genes in natural and clinical environments. In addition to metals, other toxicants are implicated in the co-selection of antibiotic resistance, including quaternary ammonium compounds and antifouling agents and detergents (Seiler and Berendonk, 2007). Therefore, the objective of this study was to determine heavy metals and antibiotic resistance of sewage bacteria, and studying the interaction of bacterial isolates with plant seed grown in the presence or absence of heavy metals to exploit.

MATERIALS AND METHODS

Isolation of Bacteria

The sewage water samples were collected from the local areas of Lahore city, Pakistan. The samples were collected in sterile plastic container and by taking all precautionary measures. The bacterial isolates were screened on Nutrient (N) agar plates supplemented with 100 µg/ml concentration of $K_2Cr_2O_7$ by the standard spread plate method. Plates were incubated at 30°C for 5 days and colonies differing in morphological characteristics and more tolerant towards the high concentrations of heavy metal stress were selected and used for further studies.

Morphological and Molecular Identification of Sewage Bacteria

For the morphological examination of selected sewage isolates, the shape, size, color and growth pattern was used to select distinct bacteria. Gram staining was done to identify Gram reaction of the bacteria. While the molecular identification of bacteria based on 16S rRNA gene sequence was done commercially. For this, the bacterial isolates were sent in Nutrient agar slants to 1st Base Laboratories, Malaysia; genomic DNA extraction, PCR amplification and sequencing were all conducted at the facility. The results obtained were in the form of identified bacteria along with their phylogenetic analysis. The sequences were analyzed using BLAST search of the GenBank Database, and the bacteria were identified based on the highest score and similarity percentage (Altschul et al., 1990).

Determination of MIC and MBC of heavy metals:

Maximum resistance of the isolates was evaluated against increasing concentrations of Cr, Cd, As, and Pb on N-agar until the strains failed to grow on the stressed media. For this, increasing concentrations of $CdSO_4$, $NaAsO_2$, $Pb(NO_3)_2$ and $K_2Cr_2O_7$ were provided in Nutrient broth medium (100-1000 µg/ml). The bacteria were inoculated and grown overnight in the stressed medium. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was evaluated by identifying concentration showing no visible bacterial growth and plating an inoculum from those stressed media tubes on simple nutrient agar at 37°C for 2-5 days. Stressed media showing no bacterial growth were plated on non-stressed media to determine MIC and MBC, where appearance of bacterial growth on non-stressed media was taken as MIC and no growth indicated MBC.

Determination of combined heavy metal effect on bacterial growth:

The combined effects of heavy metals on selected bacterial strains was determined on N-agar medium containing 50 µg/mL stress of each four heavy metals . The growth of bacterial strains was observed after 3 days of incubation.

Antibiotic susceptibility test:

Susceptibility to antibiotics was determined on Mueller Hinton agar (SIGMA) plates. Inhibition zone was noted after 48 hours of incubation. Strains were considered susceptible according to performance standards for antimicrobial susceptibility testing, obtained from the Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, PA USA 19807. The following antibiotics were tested: Amikacin (30 mcg), Colistin (10 mcg), Cefotaxime (30 mcg),

Ciprofloxacin (5 mcg), Oxacillin (1 mcg), Penicillin G (10 U), Nitrofurantoin (300 mcg), Chloramphenicol (30 mcg), Neomycin (30 mcg). Tests were performed in triplicate and zones of inhibition (cm) were averaged for the tested antibiotic for each bacterial strain.

Determination combined effects of heavy metals and antibiotics:

Combined effects of heavy metals and antibiotics on the bacterial strains was tested on Muller-Hinton agar medium, which was also supplemented with all four heavy metal stress (50 µg/mL each). The change in inhibitory zones (cm) were observed and compared with previously tested antibiotics. Test were performed in triplicate and the values were averaged.

Determination of Effect of Heavy Metals and Bacteria on Plants

The effect of heavy metals and bacteria was observed on seed germination of wheat plants. For this, bacterial inoculum was prepared by suspending overnight grown bacterial cells in 0.03 M $MgSO_4$ to a cell density of 108 cfu/ml. The seeds were washed, surface sterilized (15% H_2O_2 for 5 minutes), and again washed (5 times) with sterile distilled water. The seeds were then bacterized by dipping in prepared cell suspension for 1 hour. Control seeds were dipped in 0.03 M $MgSO_4$ for same amount of time. The treated seeds were sprouted in dark for 7 days and the resulting roots were measured for comparison between treatments.

RESULTS

Isolation of Bacterial Strains

In the present study, heavy metal resistant sewage bacteria were isolated from sewage. Three hundred colonies were screened from initial level of heavy metal supplemented N-medium. 20 isolates were selected in the secondary screening from sewage water. Finally three strains were selected based on high degree of heavy metal resistance and were used for further studies. The strains MHR-1 was Gram negative while MHR-2 and MHR-3 was Gram positive, rod shaped bacteria.

Molecular Identification

Consensus sequences of the Chromium-resistant isolated strains MHR-1, MHR-2, MHR-3 were compared with those deposited in GenBank by using the BLAST program. For phylogenetic analysis, 16S rRNA sequences of the MHR-1, MHR-2, MHR-3 and related sequences retrieved from GenBank were aligned with ClustalX v2.0 included in the MEGA5 software package (Fig. 1). All these results were qualified by using BioEdit v7.2 software. Hence, MHR-1 was identified as Escherichia coli strain, MHR-2 as Bacillus subtilis, and MHR-3 as Exiguobacterium aurantiacum strain MHR-3.

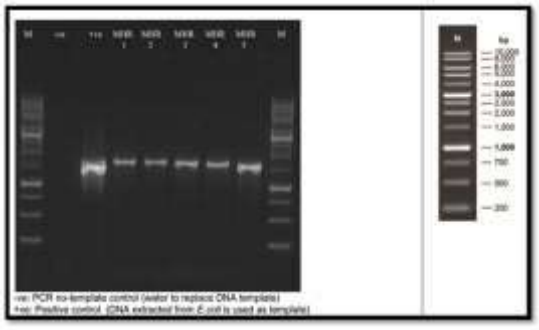

Fig 1: Results of PCR amplified 16S rDNA (performed by 1st Base Laboratories, Malaysia) for MHR-1, MHR-2 and MHR-3 in the form of bands with positive and negative controls. From left to right: Lane 1, Marker 100kb (M); Lane 2, negative control (-ve); Lane 3, positive control; Lane 4-6, Samples MHR-1, MHR-2 and MHR-3.

Heavy Metal Tolerance of Bacterial Strains

The test indicated that among four experimented heavy metals (Cr, Cd, As, Pb), maximum tolerance was shown against chromium, and isolates grew up to 600 µg/mL and minimum tolerance to cadmium showing no growth above 200 µg/mL. In general the growth rate of the MHR-1, MHR-2 and MHR-3 in the presence of heavy metal was consistently slower than the control.

MIC and MBC of Isolates

Sewage bacteria MHR-1, MHR-2 and MHR-3 showed very high degree of resistance to all heavy metals, with MIC values varying

concentration from 100-500 µg/mL and MBC values varying concentrations from 200-600 µg/mL. Among the four heavy metals tested, Strain MHR-2 was most resistant bacteria, where highest resistance was against Chromium (500 µg/mL) followed by Lead, Arsenic and Cadmium (100 µg/mL). Strain MHR-1 was also most resistant to Chromium and Lead (500 µg/mL) followed by Arsenic and Cadmium (100 µg/mL). Strain MHR-3 was least resistant to metal stress among the three bacteria tested, and showed highest resistance to Chromium (300 µg/mL) followed by Lead, and least tolerant to Arsenic and Cadmium (100 µg/mL). Moreover, Cadmium was found to be one of the most toxic metals among all the four heavy metals tested, with MBC value of 200 µg/mL against all the strains tested (Table 1).

Table 1: MIC and MBC of MHR-1 for all tested heavy metals.

Sr. #	Heavy Metal	MIC (µg/mL)			MBC (µg/mL)		
		MHR-1	MHR-2	MHR-3	MHR-1	MHR-2	MHR-3
1.	Chromium (Cr)	400.0	500.0	300.0	500.0	600.0	400.0
2.	Arsenic (As)	200.0	200.0	100.0	300.0	300.0	200.0
3.	Cadmium (Cd)	100.0	100.0	100.0	200.0	200.0	200.0
4.	Lead (Pb)	400.0	300.0	200.0	500.0	400.0	300.0

Multiple Heavy Metal Tolerance

All three bacterial strains showed growth when given a combined stress of all four heavy metal at 50 µg/mL of each metal in the media. This indicated that bacteria were able to handle multiple heavy metal stresses.

Antibiotic Susceptibility of Heavy Metal Resistant Strains

On the basis of effect of antibiotics towards microbes, inhibitory zones were measure and compared with performance standards for antimicrobial susceptibility testing. For the antibiotics studied, MHR-1 was resistant to 66% of the antibiotics tested (Amikacin, Colistin, Cefotaxime, Penicillin G, Nitrofurantoin and Neomycin), Susceptible to 22% (Ciprofloxacin, Oxacillin) and was intermediate for Chloramphenicol (12%). MHR-2 was also resistant strain to 66% of tested antibiotics (Colistin, Ciprofloxacin, Oxacillin, Nitrofurantoin, Chloramphenicol and Neomycin), Susceptible for only 12% (Penicillin G) and showed Intermediate zones against 22% (Amikacin and Cefotaxime). Strain MHR-3 showed the least resistance to all the antibiotic tested, and was resistant towards 22% of tested antibiotics (Nitrofurantoin and Neomycin), gave Intermediate zones to 33% (Cefotaxime, Ciprofloxacin and Oxacillin) and Susceptible to 45% of antibiotics (Amikacin, Colistin, Penicillin G, Chloramphenicol). Overall, results indicated that all the isolates have become completely resistant towards Nitrofurantoin and Neomycin. Additionally, the isolates that gave intermediate zones of inhibition indicate that the strains are also becoming resistant towards those particular antibiotics (Table 2).

Table 2: Antibiotic Susceptibility Results of Three Heavy Metal Resistant Strains.

Sr. No.	Antibiotic	Antibiotic Susceptibility		
		MHR-1	MHR-2	MHR-3
1	Amikacin (AK)	Resistant	Intermediate	Susceptible
2	Colistin (CT)	Resistant	Resistant	Susceptible
3	Cefotaxime (CTX)	Resistant	Intermediate	Intermediate
4	Ciprofloxacin (CIP)	Susceptible	Resistant	Intermediate
5	Oxacillin (OX)	Susceptible	Resistant	Intermediate
6	Penicillin G (P)	Resistant	Susceptible	Susceptible
7	Nitrofurantoin (F)	Resistant	Resistant	Resistant
8	Chloramphenicol (C)	Intermediate	Resistant	Susceptible
9	Neomycin (N)	Resistant	Resistant	Resistant

Combined Effects of Antibiotics And Heavy Metals on Bacterial Strains

With a combined heavy metal stress at low concentration (50 µg/mL for all hour heavy metals tested), antibiotics mostly gave synergistic effect, apparent from increased zone of inhibition size. Most of the antibiotics used in the present study showed increased effects towards all bacterial strains. Only Chloramphenicol suffered decrease in effect when tested with heavy metals towards all three bacterial strains (Fig 2). Neomycin (N) showed the most noticeable increase in the inhibition zone. In the case of strain MHR-1, all antibiotics showed the synergistic effects with heavy metals except for

Oxacillin that showed no changes in its zones of inhibition. For strain MHR-2, study showed that Amikacin and Neomycin showed the most noticeable synergistic effects with heavy metals. Again, Oxacillin and Penicillin G showed no difference in inhibition zone. Strain MHR-3, showed most noticeable results for Amikacin and Colistin where there was an increase of 0.2 cm in the zone of inhibition. Again, Oxacillin showed no change in activity in the presence of heavy metals. Overall, for all the three straisn tested, about 78% antibiotic showed increase in activity in the presence of heavy metal stress, about 11% showed no change, while the remaining 11% showed decrease in activity against the tested strains (Table 3).

Fig 2. Zones of inhibition produced by Chloramphenicol (C), Penicillin G (P) and Nitrofurantoin (F) against MHR-1 with and without heavy metal stress. Similar results were observed for all the isolates.

Table 3. Combined effects of antibiotics and heavy metals three bacterial isolates determined by measuring the zones of inhibition produced by individual antibiotic with (HM) and without (No HM) heavy metals.

Sr. #	Antibiotics	Zone of inhibition					
		MHR-1		MHR-2		MHR-3	
		No HM	HM	No HM	HM	No HM	HM
1	Amikacin (AK)	1.0	1.1	1.5	1.6	1.8	2.0
2	Colistin (CT)	0.9	1.0	0.1	0.2	1.1	1.3
3	Cefotaxime (CTX)	0.8	0.9	1.6	1.7	1.9	2.0
4	Ciprofloxacin (CIP)	2.3	2.4	1.1	1.2	2.0	2.1
5	Oxacillin (OX)	1.3	1.3	0.8	0.8	1.2	1.2
6	Penicillin G	1.0	1.2	2.1	2.1	2.1	2.2
7	Nitrofurantoin (F)	0.2	0.3	0.1	0.4	0.2	0.3
8	Chloramphenicol (C)	1.4	1.0	1.2	1.0	2.0	1.9
9	Neomycin (N)	1.3	1.7	0.9	1.3	1.8	1.9

Effects of Bacteria and Heavy Metals on Plant Growth

Effects of three bacterial strains were tested on wheat seed germination in the presence and absence of heavy metal stress. All heavy metals in concentration of 100 µg/ml showed the inhibitory effects on wheat seed growth. While the bacterial strains showed no significant effects on the seed growth in the presence or absence of heavy metal.

DISCUSSION

In this study, heavy metal tolerant strains were isolated and characterized from domestic wastewater. Three bacterial strains (MHR-1, MHR-2 and MHR-3) were selected on the basis of resistance to chromium and identified using 16S rRNA sequence analysis. Isolates were identified as Escherichia coli strain MHR-1, *Bacillus subtilis* strain MHR-2 and *Exiguobacterium aurantiacum* strain MHR-3, and all were able to grow at high concentrations of heavy metals. All the isolates exhibited high tolerance to heavy metals with minimum inhibitory concentration (MIC) for heavy metals ranging from 200 µg/mL to 500 µg/mL. Additionally, all bacterial strains showed tolerance towards combined heavy metal stress (Cr, Cd, As, Pb) of 50 µg/mL. Since the heavy metals are all similar in their toxic mechanism, multiple tolerances are common phenomena among heavy metal resistant bacteria (Mustapha and Halimoon, 2015). The isolation of bacterial species from metal contaminated environment would represent an appropriate practice to select metal resistant bacterial strains that could be used for heavy metal removal purposes (Velasquez and Dussan, 2009).

Some bacterial genera like *Bacillus* spp., *Pseudomonas* spp., *Micrococcus* sp. and *E. coli* could tolerate Cr (VI) (500 µg/mL) (Ezaka and Anyanwu, 2011). Furthermore *Bacillus* sp. and *S.capatis* were able to resist the highest concentration Pb (800 µg/ml), Cd (50 µg/mL) (Zahoor and Rehman, 2009). While MIC of bacteria for arsenic is (60 mM) (Bahar et al., 2012). In comparison with these isolates, present isolates showed more resistance towards Cd (200 µg/mL) and arsenic (200 µg/mL) while they did not showed as high tolerance for Cr (500 µg/mL) and Pb (400 µg/mL).

Several reports indicated a correlation between antibiotic resistance and metal tolerance (Spain and Alm, 2003). In certain cases metal tolerance mechanisms contribute to the increase in antibiotic resistance (Gupta et al., 2012). The occurrence of this phenomenon can be attributed to the clustering of these genes in the same plasmid (Pal et al., 2015). The antibiotic sensitivity of the identified bacterial strains was tested to explore this correlation and showed moderately to highly tolerance towards tested antibiotics. *Escherichia coli* strain MHR-1 showed 66% resistant, 12% intermediate and 22% susceptible towards antibiotics tested. While, Bacillus subtilis strain MHR-2 exhibited 66% resistant, 22% intermediate and 12% susceptible results against antibiotics. Similarly, *Exiguobacterium aurantiacum* strain MHR-3 showed only 22% resistance, 33% intermediate and 45% susceptible results towards the antibiotics. Additionally, selected strains were also checked for the combined effects of heavy metals along with antibiotics. Most of the antibiotics (78%) showed the synergistic effects, some (11%) showed no change in effect with heavy metals, while chloramphenicol (11%) gave the antagonistic results with heavy metals for all three bacterial strains. These results indicate that bacterial resistance towards antibiotics can be affected by the presence of heavy metals.

Further, the effects of heavy metals and the isolated bacterial strains (MHR-1, MHR-2 and MHR-3) on wheat seed germination were determined. The bacterial strains showed no significant effects on the seed germination or plant growth. Moreover, treatment with the three strains did not benefit the seed germination when seeds were stressed with heavy metals. Heavy metals greatly influence the growth activity of plants by completely inhibiting the seed growth and damaging the seed morphology (Sethy and Ghosh, 2013). The accumulation and distribution of metals in the plant tissue are important aspects to evaluate the role of plants in remediation of contaminated sites. Furthermore, role of metal resistant bacteria in enabling plants to tolerate heavy metal stress is very useful. Certain plant associated microorganisms have the ability to promote the enzymatically catalyzed precipitation of toxic metals (e.g., Cr, Se) by microbial reduction processes, which show considerable promise for phytoremediation of metal contaminated soils (Payne and DiChristina, 2006). Oves et al. (2013) reported that the inoculation of Cr reducing bacterium P. aeruginosa OSG41 onto chickpea grown in Cr6+ contaminated soils significantly decreased Cr uptake by 36, 38, and 40% in roots, shoots and grains, respectively, with a concomitant increase in plant growth performance compared with non-inoculated control. Similarly, metal resistant *Bacillus* sp. SC2b was capable of adsorbing significant amounts of metals (e.g., Cd, Pb, and Zn) and bacterial inoculation ameliorated metal toxicity through biosorption, thus exhibiting a protective effect on host plant growth (Ma et al. 2009). Actinobacteria can play very significant roles in the remediation of contaminated sites (El-Baz et al., 2015).

The present study is very useful to suggest that the possible impact of metal contaminated locations in human life may be greater than the direct consequence of the pollution. These results shows that the bacterial species isolated can be used as potential candidates for further work on of effluent containing metals like cadmium, chromium, arsenic and lead. Additionally, it is well established that there is a clear association between heavy antimicrobial consumption with a population and the frequent recovery of antibiotic resistant bacteria (Bergman et al., 2009). However, it is apparent that a range of other agents might represent important mechanisms that drive the selection of antibiotic-resistance determinants (Grave et al., 2010). Concluding all these facts concerning the heavy metal driven co-selection of antibiotic resistance, metals such as Cd, Cr, As and Pb are of great importance in water and soil environments that are influenced by agriculture and aquaculture. These metals are moderately to highly toxic to bacteria; they reach the environment and they accumulate to selective concentrations. Additionally, they can trigger co-selection of antibiotic resistance because responsible co-selection mechanisms that mediate resistance to these heavy metals and clinically as well as veterinary relevant antibiotics have already been described. Therefore, the elimination of antibiotics from the list of animal feed additives as growth promoters was a step in the right direction.

The present work indicates a rise in the multiple stress tolerant bacteria in sewage water which can withstand variety of stresses to ensure their survival. Massive increase in industrialization,

heavy reliance on heavy metals containing products and unchecked use of antibiotics is contributing to the origination of super bacteria that can survive well under stress. This is of great concern to human safety as these newly emerging super bacteria can create havoc if they continue to evolve in to more resistant strains that can even tolerate the most toxic compounds like heavy metals. Furthermore, rise in antibiotic resistance is taking us in to an era where antibiotics are totally ineffective in treating infections, and we need to look for alternatives that can target such super bacteria.

CONCLUSION

To conclude, sewage water is suitable for the isolation of heavy metal resistant microorganisms. Considering the wide range of multiple-metal resistance at high concentrations by the isolates, they may help in the effective bioremediation of heavy metal contaminated sites. Antibiotic resistance of non-pathogenic strains in domestic sewage water is also increasing. Moreover, the rise in antibiotic resistance in bacteria that are commonly isolated from environmental sources is a grave concern to human safety, and requires special consideration to look for effective alternatives to target such super bacteria. The present study proposes a strategy based on the use of heavy metals in low concentrations to increase the effects of antibiotics against the resistance mechanism of bacteria. A deeper understanding of the underlying mechanism of this strategy is needed to fully exploit this opportunity to target emerging multi drug resistant bacteria.

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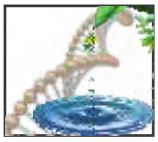

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Industrial Applications of Pectinases

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ABSTRACT: *Application of pectinases in the commercial sector has been employed for nearly a century and due to the wide range of functions that these enzymes can work for, are making them critically important from industrial point of view. Pectinases are used in the industry on their role in the degradation of pectic substances aiding and enabling in overcoming the problems faced during the processing of purees, coffee and tea fermentation, fruit juices and in other food industry related manufacturing procedures. They break down the pectin content in the plants converting them to simpler molecules of galacturonic acid. The pectinases not only help in the food industry but also have remarkable applications in the textile, including retting, degumming, bio-scouring, maceration of plant tissues, paper making, and also has role in waste water treatment. Some of the roles of these pectinases solely and in conjunction with other enzymes e.g., amylases, xylanases, cellulases etc. have been comprehensively summarized in this review.*

Key Words: *Pectinase, degrading enzymes, industrial applications, xylanases, cellulases*

INTRODUCTION

The pectinases are delicate enzymes with three dimensional structures, (Fig 1) responsible for pectin hydrolysis into simpler sugar and galacturonic acid that is poly-galacturonic into mono-galacturonic acid. It is used for a row of enzymes, as a collective term, that is involved in the breaking of pectin (Kittur et al., 2003). According to Batten et al. (2007), out of overall manufacturing of enzymes 10% of production is occupied by the pectinases. Pectinases have been produced by many microorganisms (Sharma and Sathyanarayana, 2012; Sharma et al., 2013; Mohamadi et al., 2014). The enzymes are produced by microbes in combination with other enzymes (Kaur et al., 2011; Singh et al., 2015). The microorganisms are considered as primary source of industrial enzymes in which 50% being the fungi and yeast, 35% bacteria and 15% remaining have plant and animal origin (Anisa and Girish, 2014).

The plants have cell wall which provides an informidible barrier for any foreign invasion or infection. The cell wall has an integral part or component called "Pectin" (Torres-Favela et al., 2003). It is composed primarily of galactouronans. The function of pectin is to cross link hemicelluloses and cellulose fibers, as a result of which rigidity of the cell wall is attained. It acts as a reinforce substance and is widely present in cereals, fruits and vegetables (Sathyanarayana and Panda, 2003). It is composed of modified sugars, galacturonic acid and carboxyl groups are esterified by methyl. Its major component is D-galacturonic acid (Aneeja, 1996). Its highest concentration in the plant cell wall is present in the middle lamella and has a function of strengthening substance as described by Raju and Divakar. (2013). It is a complex polysaccharide having colloidal acid with backbones of galacturonic acid residue linked by ά-(1-4) linkages. The side chains consist of galactose, xylose, arabinose and L-rhamnose (Galiotou-Panayotou et al., 1993). Pectic substances on the basis of modification of the backbone chain are classified as: Protopectin, pectic acid, pectinic acid and pectin.
Degradation of the cell wall of a healthy plant due to invasion or infection by microbes could lead to devastating effects such as cell necrosis and tissue

maceration. The diseased and dead plants due to ecological changes are recycles back to their nutrient/ elemental level by microbes that have the ability to degrade the pectin component by producing globular proteins or enzymes (extracellular and intracellular) known as pectinases.

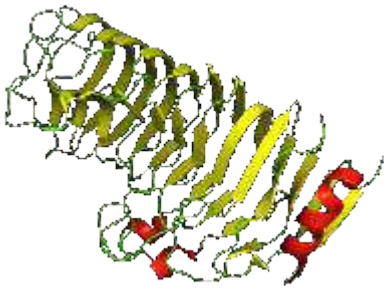

Fig 1: Computerized generated Image of polygalacturonases from Aspergillus aculeatus (1IA5) at pH 8.5 (Woo Cho et al., 2001).

SUBSTRATE OF PECTINASE ENZYMES

The compounds on which the pectinolytic enzymes act are named using the generic name "Pectic substances". These substances are acidic, negatively charged polysaccharides found in the kingdom Plantae. They are a major component of middle lamellae and are found in between the cells in the form of calcium pectate and magnesium pectate.

Pectic substances are complex macromolecules linked glycosidically having high molecular mass and are found in higher plants. They are prominently seen in the middle lamellae (also seen in the primary cell wall), and are responsible for cohesion and structural integrity of tissues present in the plant body (Rombouts and Pilnik, 1980; Alkorta et al., 1998). Some of the pectic substances are soluble in warm water while the remaining might be solubilized upon boiling in dilute acids; oxalic acid in particular, or in alkaline EDTA, or ammonium oxalates (Zhbankov et al., 1976). Precipitation of pectin can be done by using ethanol from its aqueous solution to form a gel. Pectic substances are extractable from parenchymatous and meristematic tissues. These tissues comprise of 15-30% of cell wall material while the quantity is only 0.5-1.5% for heavily lignified tissues. The lignification begins in the primary cell wall and latter extends outwards to the lamella and inwards into the developing secondary cell wall (Zhbankov, 1964).

Their presence in the middle lamellae has been confirmed by the uptake of Iruthenium red by known Pectic substances, (Sterling, 1970) and also by the estimation of pectin by using alkaline hydroxylamine (Gee et al., 1959).

Table 1 shows the relative molecular mass of pectic substances ranging from 25 to 360 kDa (Sakai et al., 1993). Fogarty and Ward in 1972 reported the pectin content of some fresh fruits and some dried plant parts, a summary of which is given in the Table 2.

Pectic substances are complex colloidal acid polysaccharides having in its structure a backbone with side chain where galacturonic acid residues linked by a-linkages constitute the backbone while side chains of pectin molecules consist of arabinose, galactose, L-rhamnose and xylose. The carboxyl groups of acid are partially esterified via methyl groups and can be partially or completely esterified by sodium, ammonium and potassium ions (Be Miller, 1986).

Pectic substances are labile which can hamper the studies of its primary structure. Extraction of these substances even in the mild conditions from the cell wall can result in artefacts that is fragments of complex compounds of polysaccharide and glycoprotein nature rather than in native molecules (Zhbankov, 1964). Dicotyledonous plants contain primary cell walls having 35% of pectic substances, but especially rich in this material are the (intercellular) middle plates.

Table 1: Molecular weights of some pectic substances(Sakai et al., 1993).

SOURCE	MOLECULAR WEIGHT (kDa)
Apple and lemon	200-360
Pear and prune	25-35
Orange	40-50
Sugar beet pulp	40-50

Table 2: List of some fresh and dried fruits and plant parts having pectin content (%) (Fogarty and Ward, 1972)

MATERIALS	PERCENTAGE OF PECTIN AS CALCIUM PECTATE	
	FRESH FRUITS	DRIED PLANT PARTS
Apples	0.5-1.6	--------
Apricots	0.7-1.3	--------
Bananas	0.7-1.2	--------
Citrus peels	--------	30-35
Currants	0.9-1.5	--------
Guavas	0.7-1.5	--------
Grapes	0.2-1.0	--------
Lemon peel	--------	35.5
Lemon pulp	--------	32.0
Lemon rind	--------	20.0
Pineapple	0.3-0-6	-------
Peas	0.5-0.8	-------
Peaches	0.3-1.2	-------
Potatoes	--------	2.5
Strawberries	0.6-0.7	-------
Sugar beet	--------	20-30
Tomatoes	0.2-0.5	-------

PECTIC POLYSACCHARIDE GROUPS:

There are several types of pectic substances (Glegg et al., 1974). These include:

1. Rhamnogalacturonans-1
2. Rhamnogalacturonan- 2
3. Homogalacturonans
4. Arabinans
5. Galactans
6. Arabinogalactans
7. Apigalacturonan

This clearly states that pectic substances have various forms in the plant tissues and this account as a probable reason for presence of various forms of pectinolytic enzymes (Jayani et al., 2005).

A committee that was appointed by the American chemical society in 1994 defined pectic substances as of the following 4 types: (Kilara, 1982; Alkorta et al., 1998).

1) Protopectin
2) Pectic acid
3) Pectinic acid
4) Pectins

TYPES OF PECTIC SUBSTANCES

1. Protopectin: These are water insoluble substances, from which soluble substances are produced, also referred to as parent pectic substance and yields pectin and Pectinic acid on restricted hydrolysis (Kilara, 1982).

2. Pectic acid: These are galacturonons containing negligible amounts of methoxyl groups. Normal and acidic salts of pectic acid are called pectates.

3. Pectinic acid: These are the galacturonons with variable amounts of methoxyl groups (>0 and 75%). Pectinates are normal or acid salts of Pectinic acid (Kilara, 1982).

4. Pectins: (Poly-methyl galacturonate): They are the polymeric materials, containing at least 75% of carboxyl groups of galacturonate units (Fig 2). Pectins are versatile, structural polysaccharides of plants. They are most prominently seen in primary cell wall and the middle lamella and occupies one-third of the dry weight of plant tissues (Gupta et al., 2008) Pectin as a plant component is present in non-woody parts of plant. Firmness and structure of plant tissues is acquired due to presence of pectin (Gummandi and Kumar, 2006). Pectin binds to the cellulose in the cell wall hence confer rigidity of the cell wall (Fig 3).

"Pectins" is the generic name used for the mixture of widely differing compositions containing Pectinic acid as a major component. In native form, it may be interlined along with other structural polysaccharides and proteins to form Protopectin (insoluble and located in the cell wall). It can be divided into 2 regions "smooth regions" and "hairy regions" (Fig 4). The source accounts for varying degree of esterification. It consists of 3 structurally well characterized motifs: HG, RG-1 and RG-2. They collectively form a network having potential for modulation in the structure as these degrading enzymes act on the cell wall.

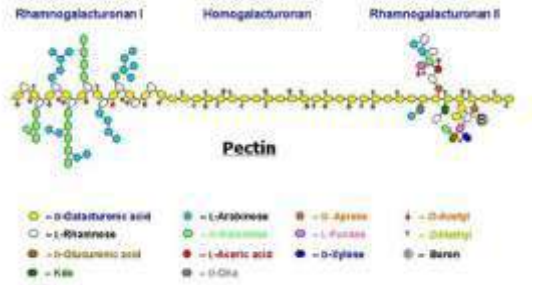

Fig 2: Structure of pectin (Harholt et al., 2010)

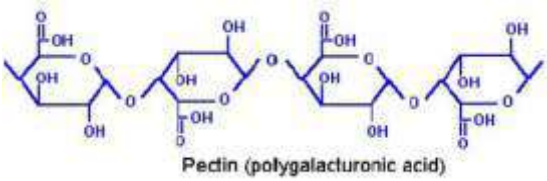

Fig 3: Structure of Pectin (Harholt et al., 2010)

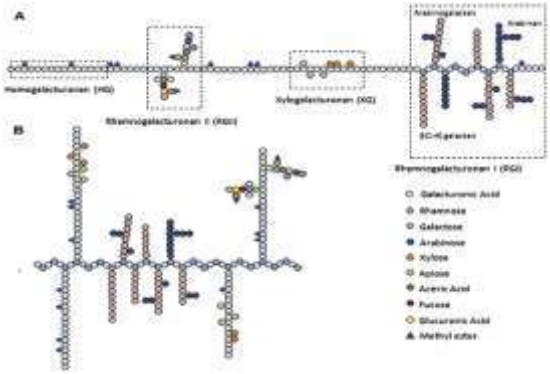

Fig 4: Schematic representatives of (A) conventional and (B) recently proposed alternative structure of pectin (Willats et al., 2006).

Pectinase Production

Pectinase as mentioned is produced and secreted by many plants and microorganisms. The list of organisms given below are examples in which PEs have been observed. (Table 3 & Table 4 given are compiled from article by Jayani et al. (2005).

USES OF PECTINASES

The functional applications of these enzymes in fruit juice and vegetable processing industries and other related industries has increased in the recent decade and more detail study on the pectinases and its wide applications has been reported by many research groups throughout the world highlighting them to be of importance

Table 3: Summary of a few reported microorganisms producing pectinolytic enzymes have been listed.

S.No	Microbial Species/ Strains	Reference in articles
1.	*Rhodotorulla sp*	(Vaughan et al., 1969)
2.	*Saccharomyces cerevaisae*	(Gainvors et al., 1994)
3.	*Phytophthora infestans*	(Forster, 1988)
4.	*Erwinia Chrysanthemi*	(Pitank and Heikinheimo et al., 1992)
5.	*Aspergillus niger*	(Maldonado et al., 1994) (Maldonaldo and Saad 1998)
6.	*Pseudomonas Solanacearum*	(Schell et al., 1994)
7.	*Lachnospira pectinoschiza*	(Cornica et al., 1994)
8.	*Penicillium frequentans*	(Kawano et al., 1999)
9.	*E.chrysanthemi 3604*	(Laurent et al., 2000)
10.	*Penicillium occitanis*	(Hadj-Taieb et al., 2002)
11.	*A. japonicas*	(Semenova et al., 2003)
12.	*Lactobacillus sub specie cremoris*	(Karam and Blarbi, 1995)

Table 4: List of plants that are able to produce pectinolytic enzymes.

S.No	Plants	References
1	*Vitis vinifera*	(Corredig et al., 2000)
2.	*Citrus sp*	(Arias and Burns, 2002)
3.	*Pouteria sapota*	(Arenas-Ocampo et al., 2003)
4.	*Carica papaya*	(Fayyaz et al., 1993)
5.	*Lycopericium esculentum*	(Warrilow et al., 1994)
6.	*Prunus malus*	(Maldonado and Evans, 1996)
7.	*Malphigian glabra L.*	(Assis et al, 2004)

(Toushik et al., 2017). Following are some uses of pectinases in the commercial sector.

1. Textile processing:

The use of different kinds of chemicals was usually observed in many wet processes in the textile industry which caused pollution and also was corrosive to cause damage. Its use was of major concern but with the introduction of use of enzymes after isolation from several sources that can serve as an ecofriendly tool. Today, enzymes are considered as an integral part of the industry. Enzymes being employed in the textiles should have the following properties:

a) Enzymes that accelerate the rate of reaction by lowering the activation energy and acts as a catalyst. i.e., remains intact at the end of the reaction.

b) Enzymes that operate under mild condition i.e., should have an optimum temperature and pH at

which it works best, while at both ends of the optimum temperature it gets degraded.

c) They should serve as an alternative to toxic, corrosive and polluting chemicals capable of eliminating the chance of causing any kind of carcinogenesis.

d) They should have high degree of specificity for substrate and its activity should be easy to control.

e) They should be biodegradable to allow proper prevention from pollution (Mojsov, 2012).

Pectinases can be used in the textiles as they can remove the cellulosic impurities from the fiber without causing any negative side effects on cellulose degradation (Hoodal et al., 2000). Applications of pectinases along with some other enzymes is used in fading of denim and non-denim, bio scouring , bio polishing , wool finishing peroxide removal, de-colorization of dyestuff etc. Enzymes have been tried in every step of wet processing including in the treatment of effluent. Example of such enzymes can be given as in bio scouring or bio preparation using pectinases that target the no cellulosic impurities within the fabrics (Lu, 2005). It is an eco-friendly tool for removing the non-cellulosic impurities from fiber with enzymes (Parveen and Suneetha, 2014).

2. **Bio scouring of cotton fiber:**

While fabrics are made from cotton the threads are coated with some adhesive substance that prevents the breaking of threads during the weaving. This is known as "sizing" and different kinds of compounds are used as a sizing agent. Starch due to being cheap was used as sizing agent. The removal of sizing agent is necessary before the fabric is processed further for dyeing. The de-sizing involves use of different chemicals such as alkali, acids and oxidizing agents. But their use can be destructing for the cotton as it not only removes the sizing agents but also degrades the fiber resulting in imperfection in dyeing and damage to soft feel of cotton. Pectinases are used in combination with other enzymes in the textile processing and has successfully been used to minimize the effects that were earlier seemed to hinder during the processing. The pectinases along with amylases, cellulases, and hemicellulases when used removes the sizing agents in a safe and ecofriendly manner and has replaced caustic soda that was earlier used for the purpose. Studies on the bio scouring of cotton using acidic and neutral pectinases have been conducted (Pusic et al., 2015).

3. **Clarification of juice and wines:**

Pectinases contribute in removing the cloudiness from juices along with enhancement in the quality and flavour of the juice. There is an increasing in the preparation of such immobilized enzymes which can be employed in the clarification and depectinization of fresh juice to overcome the problems faced in the commercial processing (Cerreti et al., 2017). Pectinases are employed in industry for juices extracted from apple, orange, lemon, guava, grapes and many other fruits, use of pectinases in these fruits have been explained here briefly.

a) **Orange juice:**

The pectin found in the orange juice gives it the particular appearance. Clarification of these substances is necessary to make them marketable and presentable on commercial level. The clearing of the pectic substances by degradation not only make the juice visibly clear but also aids in improvement of its yield overall. The classical methods that were been used involved heating or freezing, and were not good in respect to cost and quality of the juice that was extracted. Freezing method was expensive while heating spoiled the flavour of the juice (Braddock, 1981). The pectinases can be used in the orange juice industry to accomplish the following targets:

1) To soften peels so that peeling off of the citrus peels makes the processing of the juice easier (Ciechariska and Kazimierczak, 2006).

2) The pectinases are also applied in the crushing and clarification steps. The sediments suspended in the juice interact with the pectinase resulting in the juice being less viscous and increase yield due to the liquefaction that occurs during the

action of pectinases (Kareem and Adebowale, 2007).

They have been reported to increase volume to increase the volume of the juice (Kashyap et al., 2001).They have also been reported to impart critical role in the enhancement of the flavour of the processed juice.

b) **Lemon juice:**

` Similarly, clarification step during lemon juice processing is aided with enzymes. Pectinases not only help in the extraction of juice but also benefit with increase yield of juice which is concentrated easily to be marketed, and reduction in processing time has also been reported (Prathyusha and Suneetha, 2011). The lemon juice was traditionally extracted relying on pectin esterase which was naturally present in the fruit content. The products obtained were majorly cloudy peel products. The peels with the pulp were ground into pieces of 3-5 mm and were heated after adding water in 1:1 ratio at a temperature of 95^{o}C to destroy PME, but still the juice can have the pectic content, by using enzymes for degradation followed by centrifugation and pasteurization can process juice good enough to be concentrated and to be marketed.

c) **Apple juice:**

Apple juice can be obtained through a 2-step process i.e., firstly the treatment of the crushed apple with enzymes followed by pomace liquefaction treatment of with the pectinases and cellulases to completely extract the juice (Will et al., 2000). After washing and crushing, the apples are pressed to get the juice. The pectinases are added to facilitate juice extraction and pressing efficiency so that the mixture can be separated to be processed using sedimentation, filtration and centrifugation steps along the way to get the desired products (Fig 5). Process of production of apple juice has been shown in a comprehensive flow chart of the process. The juice is pasteurized to inactivate the enzymes in order to maintain the same cloudy texture of the fruit while centrifugation to acquire clear juice (Yamaski et al., 1964; Grassin and Fauquembergue, 1996; Kashyap et al., 2001). Polygalacturonases and pectin methyl esterase combinely are used for the proper clarification of the juice or pure pectin lyase can be employed to get clarification (Ishii &Vokotsuka, 1973).

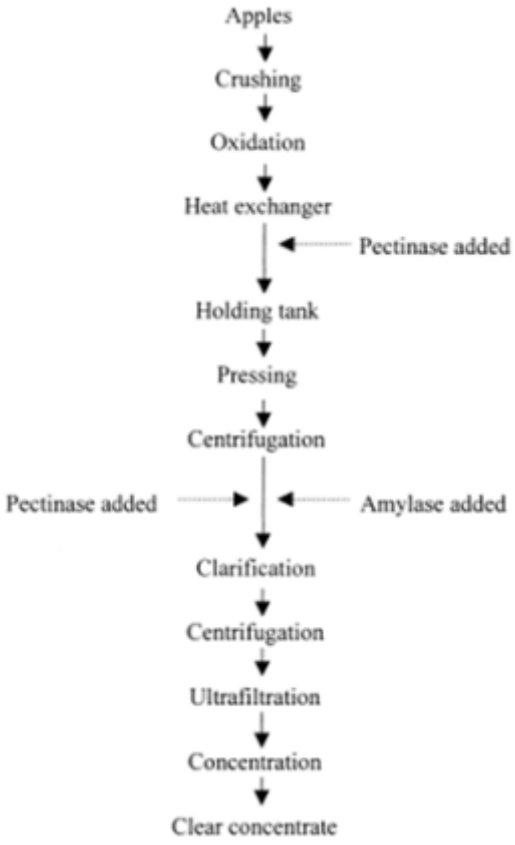

Fig 5: Flow chart showing the Production of apple juice (Grassin and Fauquembergue, 1996).

d) **Grape juice and wine making:**

Grape juice is either too sweet or too acidic in nature to be consumed in alone therefore it is consumed while mixing it with other fruit juices e.g., apple or in the form of mix fruit juices. Due to their high pectin content the grapes were earlier reported to be difficult to press and crush. The fruit after pressing and crushing was heated at 60-80^{o}C in order to allow release of colour and the running juice released (with small solid particles in it) was subjected to filtration or centrifugation. Use of several enzymes like pectinases and hemicellulases can help in reducing the haze and gelling properties of pectin, along with removal of tartarate, to reduce the overall acidity of the grape juice to a considerate

level. The juice after treatment with enzymes can be filtered and then concentrated, pasteurized and bottled for use.

The treatment of macerated fruits with enzymes (before addition of the inoculum) improves the characteristics of the wine (Parveen and Suneetha, 2014). The pectinases can be applied to the grape juice processing or wine production in the following steps as summarized in the Table 5.

Table 5: Use of enzyme in different steps of the juice/ wine processing summarized.

S.No	Step of juice/ wine processing	Enzymes used at level	Advantages	References
1	First step	During crushing	Reduced processing time, and increases volume of free juice released, juice is clarified and better release of anthocyanin.	(Pretel et al., 2000) (Pilnik and Voragen, 1970)
2	Second step	Before and after fermentation (in case of wine production)	Settles the suspended particles and removes undesirable microorganisms.	(Kashyap et al., 2001)
3	Third step	When wine is ready to be transferred and bottled.	Aid in clarity and increase filtration rate.	(Kashyap et al., 2001)

e) Extraction of juice from jackfruit, pineapple and guava:

Juices from jackfruit, guava and pineapple are turbid, viscous and grey in colour. Due to the change in the pattern of consumption of these fruits over the years in the population, has made them the alternatives to caffeine-containing beverages (Jagtiani et al., 1988; Sevda et al., 2012).

The commercial preparations of these juices require improvement in many ways as these juices can be preserved in the form of pulp/ purees. The single strength juice preservation is not economical so there is a need for alternatives to have bulk production of fruit juice without damaging the flavour and texture of the juice in the processing. The fruit juices usually contain a water content ranging from 75%-90 % (Young, 1975). So, the fruit juice needs to be concentrated in order to attain microbial stability and for transportation of bulk juice by making it economical process as weight to volume ratio is reduced when juice is concentrated. Some issues related to the taste and colour were also reported as the juice extracted was viscous and had a sufficient amount of fiber content that contributed to its cloudiness as well. The problems faced in the classical methods of processing were observed to be resolved when enzymatic liquefaction was employed to these fruits using pectinases as the enzymes for treatment (Ahmed et al., 2014). It helped in the clarification and concentration of the juices. Pectinases not only helped in increasing the volume of the juice but also contributed in the solubilizing contents, color and aroma stability and clarifying the cloudiness by degrading the fiber content in these juices (Buchert et al., 2005). The clarified juices are more attractive and are generally acceptable to the population for use and can be processed for making of other fruit products like nectar, jelly and mix fruit juice etc. A summary of extraction of fruit juices from jackfruit (Artocarpus heterophyllus), pineapple (*Ananas comosus* L.) and guava (*Psidium guajana*) using pectinases have been summarized in the following flowcharts (Fig 6).

Fig 6: Extraction of juices using pectinases in the enzymatic liquefaction process (Compiled from Ahmed et al., 2014).

f) Strawberry, raspberry and blackberry:

The juices extracted from fruits like strawberry, raspberry and blackberry have high pectin which needs to be de-pectinized in order to get clear concentrates otherwise they will appear in the juice as residues (Will and Dietrich, 1992). Some extraction of juice from these soft fruits is possible

but difficult to process.

Sometimes the pectin & hemicellulose interact with other components such as proteins and form complexes that are not easily removed by the enzymes. These berries when treated with Rapidase BE in the maceration step helps in their juice extraction. Use of pectinase if necessary, ensures the removal of any residual left in the juice, followed by its filtration and stored at low temperature and dark conditions (Kashyap et al., 2000).

4) **Preparation of purees:**

Pectinases can also be used in making purees from prunes, apricot, peaches, strawberries and fruits like mango, guava etc. the enzyme soften the skin and tissues (Tapre and Jain, 2014). The enzyme changes the fleshy pulp into semi-liquid product that is concentrated to 3 fold to form good textured puree to be sold as product. The puree produced in this way in case of guava can be used to make jams, syrups and juice blends etc. fruit pulp is treated with exogenous enzymes to yield juice. These enzymes have been reported to be used in the treatment of the pulp (Chaudhri and Suneetha, 2012; Khan et al., 2013).

5) **Boosting aroma and flavour:**

Variety in aromas depends on the variety of grapes used and form the aromatic profile of wines. The aromas also originate with the yeast fermentation (Pineiro et al., 2006; Vilanova and Sieriro, 2006; Siero et al., 2012). The wines have aromatic components as well as the volatile components. The aromatic components contribute directly towards the scent of that wine (Wiliams et al., 1989; Bayanore, 1993; Winterhalter and Skouroumouvis, 1997). Use of pectinases help in breaking down the cell walls of grapes thus extracting their aromatic precursors. The interaction with these precursors via beta-glycosidase in the must, result in the increased components, enhancing the aroma of wines (Gomez-Plaza et al., 2000; Pinelo et al., 2006; Comitini et al., 2011; du Toit et al., 2011; Siero et al., 2012).

6) **Maceration of plant tissues:**

The maceration process of plant tissues by enzymes help in transforming the tissues into suspension with cells that are intact (Bock et al., 1983) and are used in the production of pulpy products such as food including juices, baby food, nectars etc. Enzymes such as pectinases or their use in combination with cellulases and hemicellulases is reported. The enzyme that specifically targets the middle lamella (Kashyap et al., 2001), the endogenous PE needs to be inactivated as it is very important for many macerated products (Dongowski and Bock, 1980). Enzymatic maceration results in limited degradation of the pectin components. This process can be used for carrots and dried instant potato mash (Boke et al., 1979; Bock et al., 1984).

7) **Retting and degumming of plant fibers:**

Bast fibers are formed in the cortical region of plants in groups outside the pericycle, e.g., Ramie and sunn hemp. As they contain gum, it has to be removed before processing it for texture making (Hoondal et al, 2000). The bast fibers are required to be ret in order to be polished for its application in the textile sector e.g., in the production of commercial fibers. Retting is a process by which fibers in the form fiber bundles get separated from the cuticularized epidermis or woody core cells of the plant. The microbial activity in respect to the partial degradation contributes in the separation of cellulosic fibers from non-fiber tissues enabling us to extract the target resource for manufacturing, easily. Studies and work on retting of flax has clearly demonstrated the use and need of pectinases (Sharma and Van Sumere, 1992). Pectinases that are alkaline in nature are mostly used for the retting and degumming of jute, hemp, kenaff (Chesson, 1980; BruhImann et al., 1994). Retting is a process in which the pectin is decomposed by the bacteria and fungi while the fiber is released from the bark. The bacterial species from the genus Clostridium and Bacillus along with Aspergillus and Penicillium can be employed in the processing of the retting (Sharma and Robinson, 1983). Genus Clostridium species, Clostridium butyricum and Clostridium felsineum are regarded as major retting agents (Hellinger, 1953; Vonzyakovskaya et al., 1974; Kashyap et al., 2001). Pectinases have been used for retting of flax to separate the pectin from the fibers as reported by Hoondal et al. (2000). Retting of Latvian hemp sort "Purini" by use of pectinases has been reported by

Bernava. (2015). The treatment of pectinases along with xylanases is now days suggested to be economical as well as serves as the best alternative to the toxic and polluting chemicals (Kapoor et al., 2001). Study on bacterial pectinolytic enzymes, has been conducted that are used in retting and degumming of natural fibers (Chiliveri et al., 2016).

8) **Oil Extraction:**

Oil extraction from citrus peels is done to obtain the citrus oils by using pectinases to remove the pectin content in these oil seeds which can cause emulsification hindering in the extraction process (Scott, 1978). Lemon oil is extracted via use of enzymes like pectinases. The extraction of these oils is done using an enzyme only or an extraction can be prepared by combination of more than 2 enzymes. Enzymes were used to degrade the cell wall usually in the grinding step or in the liquefaction procedure. For example, Table 6 shows a few enzymes used in combination to extract oil (Kashyap et al., 2001). Oil extraction form oil seeds like canola, coconut germ, seeds of sunflower, palm, olives and kernel were earlier done by using organic solvents. These solvents were not entirely beneficial as some were potentially injurious for health, e.g., hexane used in the process was a potential carcinogen, and therefore these had to be replaced by enzymes.

Table: 6 Extraction of oil from oil-bearing material using enzymatic processing.

Oil seeds	Enzymes	Use level percent (w/w)	References
Rapeseed	Pectinase, Cellulase	**0.1-3**	(Deng et al., 1992)
Soybean	Protease	**0.2**	(Yoon et al., 1991)
Coconut	B-Glucanases, Pectinase, α-amylase and protease	**0.1**	(Barrios et al., 1990)
Avocado	α-amylase	**1.0**	(Domiguez et al., 1995)
Sunflower	(Ultazyme) cellulase,α-Galacturinidoglicano-hydrolase	**1.5**	(Lanzani et al., 1975)
Peanut	Cellulase, protease	**3.0**	(Lanzani et al., 1975)

9) **Treatment of pectic wastewater:**

The waste water usually contains pectin content when released out and this pectin is not degraded well by the microorganisms in the activated sludge treatment step. Treatment of such waste water from citrus-processing industry was reported by using alkalophillic *Bacillus* sp (GIR 621) as reported by Tanabe et al. (1987). The treatment of wastewater with this enzyme has proved to be efficient in removing the pectic substances from waste water.

A soft rot pathogen, *Erwinia* carotovora (FERM P-7576) was reported to secrete an endo-pectate lyase which was reported to be effective in the pretreatment of wastewater but due to its phyto-pathogenecity, indirect pretreatment by enzyme produced from the bacteria was compared and reported, that all the pectin was solubilized that was present within the waste water (Tanabe et al., 1986). Similarly, the treatment of vegetable food processing waste contains pectin in the form of a by-product. Pretreatment with these enzymes facilitate the removal of pectin and makes it suitable for the degradation by activated sludge treatment (Hoondal et al., 2000; Jayani et al., 2005).

CONCLUSION

Pectinolytic enzymes are produced by many organisms such as bacteria, fungi, yeasts and plants. Microbial pectinases are important in the decomposition of dead plant materials, contributing in the nutrient recycling e.g., in the carbon cycle. Microorganisms are major sources of enzymes. Microorganisms produce multiple pectinase forms which differ in their properties and molecular mass. Pectinases produced by bacteria and fungi are employed for commercial scale production of several food and textile related products. These enzymes are hydrolytic in nature and help in the degradation process majorly contributing to the food and juice processing industry. Pectinases are no doubt had their importance in past, have in present and will remain in future but there is always a need of faster, cheaper and heat stable enzymes in the industries. Therefore, more research is required to boost industrial applications of pectinases in an economical way.

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All manuscript should have a uniform style parts printed on one side of the paper, font size 12, Times New Roman, Justified, double spaced with margins of at least one inch on all sides. The manuscript should consist of following subsections (1) Title Page (2) Abstract (3) Text (to be subdivided into Introduction, Materials and Methods, Results and Discussion (4) Conclusion (5) Acknowledgement (6) References.

Manuscripts including tables, figures, illustrations and references, should be prepared in accordance with the publication Manual of the American Psychological Association (5th Edition). Tables and figures should not be embedded in the text rather insert them after references OR should be included as separate sheets or files. A short description title should appear above each table with a clear legend and any footnotes suitably identified below. All units must be included. Figures should also be completely labeled.

Title Page

The first page will be the title page which includes the following: (i) Comprehensive title of paper, (ii) Complete names of authors, (iii) institutional affiliation/s (iv) complete e-mail address of the corresponding author.

Abstract

Abstract not more than 300 words should also be on a separate page with 3 to 5 key words at the end abstract.

Introduction

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and Methods

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results and Discussions

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the past tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section. The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

Conclusion

State the conclusions at the end of the paper.

Acknowledgement

The Acknowledgments of people, grants, funds, etc should be brief.

References

In the text, a reference identified by means of an author‘s name should be followed by the date of the reference, in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by “et al.,”. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples

Smith (2000), Blake et al., (2003), (Kelebeni, 1983), (Chandra and Singh, 1992), (Chege, 1998; Steddy, 1987a,b; Gold, 1993,1995), (Kumasi et al., 2001)

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Diaz E and Prieto MA (2000). Bacterial promoters triggering biodegradation of aromatic pollutants. Curr. Opin. Biotech. 11: 467-475.

Dorn E and Knackmuss HJ (1978). Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1, 2 dioxygenases from a 3-chlorobenzoate-grown Pseudomonad. Biochem. J. 174: 73-84.

Pitter P and Chudoba J (1990). Biodegradability of Organic Substances in the Aquatic Environment. CRC press, Boca Raton, Florida, USA. 4

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Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences:

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Ethical Issues: In case of articles reporting experiments on higher invertebrates or vertebrates, author must declare that the study was approved by the institutional ethical committee. The work carried out on the human subjects must include that the consent was taken from all respective subjects.

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