To Study the Effect of Plasma Treatment on Fungal Decontamination in wheat and Biochemical Parameters Thereof

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Thesis submitted
in partial fulfilment of the requirement of the degree
of
Master of Technology
In
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CERTIFICATE

This is to certify that the thesis entitled **"To Study the Effect of Plasma Treatment on Fungal Decontamination in wheat and Biochemical parameters Thereof"** being submitted by **Ms.Nisha Mahey** (Roll No-601404013) in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of **Dr. Sanjai Saxena** (Internal Supervisor) and **Dr. Sunita Mishra** (External Supervisor) CSIR-CSIO and that no part of this thesis has been submitted for the award of any other degree.

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I hereby declare that the work being presented in the thesis entitled **"To Study the Effect of Plasma Treatment on Fungal Decontamination in wheat and Biochemical parameters Thereof"** in partial fulfilment of the requirements for the award of degree of Master of Technology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of **July 2015** to **Dec 2015**, under the conception and supervision of **Dr. Sanjai Saxena**, Professor, Department of Biotechnology, Thapar University, Patiala and from **Jan 2016** to **Jun 2016** under the conception and supervision of **Dr. Sunita Mishra**, Head Principle Scientist, CSIR-CSIO. I have not submitted the matter embodied in this thesis for the award of any other degree.

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I dedicate this thesis to my beloved family who are an ever supporting and encouraging with their great patience.

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ABBREVIATIONS

S.no	Abbreviation	Full form
1.	$AlCl_3$	Aluminium Chloride
2.	APS	Ammonium Sulfate Per
3.	BOD	Biological Oxygen Demand
4.	BSA	Bovine serum albumin
5.	CFU	Colony forming Unit
6.	FRAP	Ferric Reducing Antioxidant Power
7.	FTIR	Fourier Transform Infrared Spectroscopy
8.	H_2SO_4	Sulfuric acid
9.	KBr	Potassium bromide
10.	mbar	Millibar
11.	nm	Nanometer
12.	$NaNO_2$	Sodium Nitrite
13.	NaOH	Sodium hydroxide
14.	psi	Pounds Per Square Inch
15.	ppm	Parts Per Million
16.	PAGE	Polyacrylamide gel electrophoresis
17.	RPM	Revolutions Per Minute
18.	SDS	Sodium dodecyl sulfate
19.	UV-Vis	Ultraviolet visible
20.	V	Voltage

Executive Summary

In the present study the effect of plasma treatment for surface sterilization and microbial decontamination purposes were studied. Wheat (*Triticum aestivum*) variety HD3086 was choosen as it is among the major crop being consumed in Northern Hemisphere. The wheat grains were infected with storage fungus *Aspergillus flavus* (MTCC 817), which is among the most potent fungus. The aflatoxins produced by this fungus causes very deleterious effects. The major problem caused by storage fungi are post harvest losses. Main objective of our study was to diminish the deterioration of crops and grains every year, which helps in reducing a vast economic loss. The SF_6 gas was used in plasma reactor for fungal decontamination. The effect of SF_6 gas was seen on fungus at different time intervals, to evaluate the minimum duration to destruct the fungal spores present. FTIR analysis was done to compare the difference between the chemical composition of plasma treated and untreated wheat sample. During application of any treatment, it is of prime importance that the treatment should not have any harmful effects, only than it will be considered as ideal treatment. To study the nutritional quality, Proximate analysis were done. Further antioxidant activity and cooking properties were studied to compare the plasma treated and untreated sample. Proximate analysis of the plasma treated sample showed reduction in moisture content value from 9.86% to 8.68%, as the presence of moisture content provides appropriate environment for fungal growth, so reduction in moisture content is an effective result. Cooking time of plasma treated sample was reduced to 6.3 min from 7.4 min which helps in preventing use of resources. Protein profiling was done to compare the Plasma treated and untreated wheat sample by SDS-PAGE. A slight increase in the antioxidant activity of wheat extract is also observed in plasma treated sample.

Keywords: Fungal Decontamination, *A.flavus*, Plasma treatment, FTIR Analysis, Cooking Properties, Proximate Analysis, SDS PAGE, Antioxidant activity.

1. Introduction

Cereal grains are considered to be major source to meet the nutritional requirements of human dietary system. Among the world population rice, wheat, and maize, are consumed at major scale and to a lesser extent, millets and sorghum are important staples required for daily survival of billions of people. Cereal grains plays an important role in transforming human civilization by domestication of major cereal crops in different regions of the world. Wheat (*Triticum aestivum* L.) is considered to be most primitive domesticated crop in the Fertile Crescent of the Near East around 10,000 years ago (Gustavasson, *et al.*, 2011).

Among all the grain crops, wheat is the most extensively grown crop (Araus *et al.*, 2007). In the Northern hemisphere wheat is consumed as an important food crop to provide source of energy through the presence of carbohydrates and essential proteins (Belderok *et al.*, 2010).

Food and agriculture organization of U.N. estimates that near about 1.3 billion tons of food crops are globally wasted per year (Gustavasson *et al.*, 2011). In present era, food safety is the crucial issue for both consumers as well as food industry. One of the major cause of post-harvest losses of food crops is fungal contamination. With increasing population, there is urgent need to increase the food production to feed people. This can be achieved by minimizing post harvest losses during seeds and grains storage. The seeds and grains storage are affected by different factors which firmly decide their keeping quality. These factors include the condition of the product before storage, storage container and the duration period for the storage of product (Sinha *et al.*, 1973). Cereals are at high risk to mould attack at the pre or post harvesting stages of agriculture crops.

During strorage, the moisture present in the product reaches an equilibrium value with air present in between the particles of product and a relative humidity level is formed that provides appropriate conditions for the development of deteriorative organisms. Temperature and moisture had great effects on the enzymatic and biological activities which further leads to rate of spoilage of stored grains.

Contamination of foods induces by fungus can causes economical and social burden on health care through the synthesis of mycotoxin (Misra *et al.*, 2011). In case of crops, toxigenic fungus are divided into field fungi, which destruct the surface of crops

by production of toxins before the harvest period, and storage fungi which infect the crops after the harvest period. Main reason for the invasion of crops by storage fungi are physical conditions such as moisture and temperatue (Miller1995). Fungal growth over crop affect the crop quality such as change in appearance, musty odors and loss in seed germination property and further leading to spoilage and germplasm damage during storage of seeds and grains. Some of the pathogenic fungi also produces aflatoxins which can cause diseases in humans and animals while consumption (Maity *et al.,* 2004). A number of storage fungi are present which are responsible for spoilage of seeds and grains but the most commonly found fungus are *Aspergillus, Penicillium* and *Fusarium* species (Reddy *et al.,* 2008; Selcuk *et al.,* 2008). Except fungus, insect invasion is a major problem which spoil the grain tegument and leads to production of carbonic acid and water because of which humidity content get's increased that favours fungus multiplication. It leads to the spread of fungal spores in the bulk grains (Santos and Mantovani 1997).

Various physical and chemical methods are being employed to combat this devastating post harvest loses of stored grains. Chemical methods include the application of chlorine solutions but its trace particles can poses serious health problems (Park *et al.,* 2008). Physical methods includes thermal and non thermal sterilization for fungal decontamination. Surface sterilization method includes seed treatment with hot water, aerated steam, commercial bleach, fungicides etc. Inactivation of pathogens can be done efficiently by thermal sterilization methods but it induces side effects in the functional and nutritional properties of food.Various other methods used for fungal decontamination are microwave irradiations, radio frequency, Ultra violet radiations, gamma radiations, low energy electrons and hydrostatic pressure (Yao *et al.,* 2005). Ultra violet radiations comes under non-thermal treatment for the bacterial decontamination without having any effect on the nutritional quality of food (Smith, Lagunas-Solar & Cullor, 2002). However, this method is very time consuming, costly and not completely effective.

Plasma technology is an emerging field for surface sterilization and pathogen decontamination. "Plasma" was first coined by Irving Langmuir and Lewi Tonks and is defined as the fourth state of matter. It is a highly energized gas composed of excited atoms and free electrons molecules, ionized gases and radicals. These intrinsic particles

results in surface etching and microbial inhibition (Moisan *et al.*, 2001). The diffusion of these reactive species in between the surface leads to various physical and chemical changes (Poncin, Epaillard, Brosse & Falher, 1999; Mozetic 2001). Plasma treatment for fungal decontaminations of various food crops is a very sparsly studied area with scanty preliminary data. Wheat deterioration is a major problem which leads to a great loss of grains every year. It is difficult to decontaminate the surface of wheat because of its structure having deep ventral furrow.

Hence, the present study was undertaken to assess the effect of cold plasma treatment on physical, biochemical and nutritional properties of Wheat.

2. Review of literature

2.1 Post Harvest Losses

A major issue in food safety are post harvest losses, a large amount of cereals, fruits, vegetables and other food material are spoiled every year during the storage process. These losses costs huge economic losses every year, as post harvest and marketing system are interconnected activities. Crops during the agriculture process undergoes various steps such as harvesting, drying, processing, storage and transportation. The major aim of the post harvest storage is to attain customer satisfaction in the field of quality, nutrition and safety. It has been evaluated that post harvest losses are less in developed countries as compare to developing country and the reason being for this is the well organized farming system, good transport infrastructure and the most important factor is efficient storage conditions (World Bank *et al.,* 2011). When the cereals and grains are stored the presence of moisture and temperature appreciate the growth of microorganisms and fungus in the stored grains, which results in rejection of these grains and cereals because of bad odor, quality reduction. The presence of fungal spores in the bulk grains leads to the production of mycotoxins which are carcinogenic (Magan *et al.,* 2004).

The three most consumable food crops in the world are wheat maize and rice. These cereal grains contributes more than half of the calories being consumed by human beings. After Rice, wheat is second important source of dietary nutrients among humans. In general, wheat cultivation is possible in wide range of environmental conditions viz. wide temperature range and water availability. Wheat is widely cultivated during the spring and winter season. China is the largest producer of wheat throughout the world, and India producing approximately 30% of world wheat (FAOSTAT, 2013).

Figure 1: Wheat production in world

2.2 Effect of Fungal Contamination on Seeds and Grains

Seeds and grains are contaminated by various microorganisms. Deterioration from natural food enzymes also occurs in food which causes deleterious effects among humans and animals. Although food industry has to undergo great economic loss because of food spoilage (Stoica *et al.*, 2011; Afshari and Hosseini, 2014). More than 25 different fungal species are reported to invade seeds and grains during storage period (Duan *et al.*, 2007). However, various species of *Aspergillus* and *Penicillium* are responsible for most of the spoilage and germ damage during storage period. They cause decrease in quality of baking and nutrition value and produce undesirable odors and change of appearance of stored food grade grains is also observed.

In addition many fungi produce mycotoxins that can cause serious health hazards among human beings and animals and make products to be rejected for edible purposes which further results in economic losses. *Aspergillus flavus* is among the most popular food borne fungi. It is well known for the production of carcinogenic aflatoxin. Industrial use and toxigenic potential of *A.flavus* has gained a worldwide attention. Fungal infestation of seed coat decrease the self-life of seeds, or may cause abnormal seed lings.

2.3 Various Strategies Used for Microbial Inactivation

Cereals are among the most important food in contribution to the nutritional value. Wheat is consumed at large scale among north region with the production rate of 713 million tons per year. A large amount of stored wheat is spoiled every year because of the presence of storage fungi, bacterial contamination and inappropriate storage conditions. To prevent this spoilage various physical and chemical methods were implemented for microbial inactivation. In conventional seed treatment compounds or processes are applied to grains in order to reduce damage by microorganism that infest the seed coat and inner part of seed. There are various methods which are being used for suppression of fungal growth and mycotoxin production. These methods are divided into three categories- Physical, chemical and Biological method.

2.3.1 Physical Methods

This method includes thermal inactivation (heat treatment), irradiation (UV, gamma, microwave etc), aeration, moisture control and modified atmosphere conditions. Irradiation was considered as the most effective method which includes UV, gamma and Microwave irradiation for food crops decontamination purposes, But these methods have some harmful effects on the crops which could not be ignored. Chlorophyll content and yield of the crops is adversely effected by UV radiations implementation (Kakani *et al.,* 2003). Gamma radiations reduces plant germination, plant life, plant height and also the grain number or grain weight (Mushtaq Ahmad Khah *et al.,* 2015). The composition and nutritional quality of legumes is adversely effect by Microwave radiations (Hefnawy *et al.,* 2011). The treatment of wheat seedlings with microwave radiations reduces their germination and vigor index (Gaurilcikiene *et al.,*2013).

2.3.2 Chemical Methods

Chemical method involves the treatment of the sample with chemical solutions like chlorine solutions (Sinha, Yamamoto, & Ng, 1997), use of KIO, azodicarbonamide, KBrO, ascorbic acid (Junqueira *et al.,* 2007), benzoyl peroxide and recently ozone treatments, and enzymatic approaches such as use of lipoxygenase, proteolytic enzymes, redox enzymes, and reducing agents in combination (Lamsal & Faubion, 2009) are being used. But the major disadvantage of these methods is that the trace elements of these chemicals imparts negative impact on the human health and animals.

2.3.3 Biological Methods

This method involves the usage of microbial strain for the removal of mycotoxin (Kabak *et al.,* 2006).

2.4 Plasma Treatment: A Decontamination Tool

Plasma is defined as highly energized gas, fourth state of matter which is composed of excited atoms or molecule, radicals and free electrons (Moisan *et al.,* 2001). The term Plasma was first coined by Irving Langmuir and Lewi Tonks. Plasma treatment application nowdays are used for a number of applications ranging from polymer textile

industries, bio-medical studies, Material processing, Semiconductor chip fabrication anticorrosion coating and a recent concept tested is sterilization and coating of the materials used in food packaging (Laroussi 2005; Lopattananon & Jones, 2000; Helhel *et al.*, 2005; Chu 2007)

Plasma technology is well known for its antimicrobial and surface engineering properties among various fields like textile, polymer industries and bio-medical. Etching, bonding of plastics, surface cleaning and sterilization are the industrial plasma processing procedures (Naebe *et al.,* 2010; Vlachopoulou *et al.,* 2009). The basic principle of plasma technology is that the gas is fed with additional energy by an electrical discharge, afterwards gas returns into gas rich plasma state. Plasma is differentiated on the basis of their energy level as Non thermal and Thermal plasma.

Cold plasma comes under non thermal technology. In this technology, the temperature of the sample surface is maintained at a temperature below to thermal treatment temperature for the inactivation of bacterial endospores, molds and yeasts, respectively under non thermal conditions (Schluter and Frohling 2014). The composition of the formed plasma rely on the used plasma source, gas used and the application (Weltmann *et al.,* 2008; Ehlbeck *et al.,* 2011). Cold plasma treatment consist of various reactive species, such as ultraviolet, photons, electrons, ions, free radicals, and atoms. The system used for the microorganisms inactivation by cold plasma treatment is the oxidation of microbial cell membranes by reactive species (Gallagher *et al.,* 2007; Laroussi and Leipold, 2004).

Antifungal efficiency of low pressure cold plasma was observed against *Aspergillus parasiticus*. Pistachio nuts, hazelnuts and peanuts were artificially contaminated with a known concentration of *Aspergillus parasiticus* spores and kept for incubation period. The gases used for the antifungal treatment was SF_6 and air gases till the duration of 20 minutes in order to detect the minimum time for decontamination. The output of this work showed that with air gas plasma half of the total aflatoxins are reduced by treating at the duration of 20 minutes. While using SF_6 Gas in the plasma treatment one fourth concentration of the aflatoxins are reduced (Basaran *et al.,* 2008). Disposable food containers are mostly preferred nowadays for food packaging. In consideration of health issues it is must that the food containers have to be clean.

Wheat deterioration is a major problem which leads to a great loss of grains every year. There was need for study to protect wheat grains from spoilage, thus inactivation of microorganisms could be achieved by Cold plasma treatment (CPT) without an increase in temperature. Instead of inactivation germination capacity of grains are also enhanced by this treatment (Fernandez *et al.*, 2011).

2.4.1 Bacterial Decontamination

Inactivation pattern of *Salmonella* sp. by using low temperature plasma was studied (Fernandez *et al.,* 2012). The factors which are responsible for inactivation of *S. Enteritidis* and *S. Typhimurium* are the higher humidity level and the oxygen presence in the plasma (Kim *et al.,* 2011; Ragni *et al.,* 2010). Cold plasma treatment is an emerging field in order to decontaminate the foods such as cereals, grains, fruits and vegetables. The effect of cold plasma treatment has been studied on various spore forming bacteria such as *Bacillus atrophaeus*, *Bacillus cereus* and *Geobacillus stearothermophilus.* This study concluded that nitrogen-cold atmospheric plasma treatment has a biocidal effect against few spores and the probable reason for this is spore integrity loss and variation in the morphology of spore surface (Van Bokhorst-van de Veen *et al.,* 2014).

2.4.2 Effect of Plasma Treatment on Biochemical Parameters of Seeds and Grains

A reduction in the rate of chronic diseases like cardiovascular and cancer were observed by the grain consumption, because of the presence of the phytochemicals present in the grains. Wheat is considered among the major crop for human diet. Different wheat varieties possess various phytochemical profiles and antioxidant property, which effects the nutritional value of wheat product and hence influence human health benefits. The main objective of the study was to compare the antioxidant property and phytochemical profiles of different wheat varieties. The value of total phenolic content, total flavonoid content and total antioxidant activity have not shown any significant difference among the 11 varieties of wheat. But a remarkable difference between the carotenoid content and total ferulic acid content was observed (Kafui Kwami Adom *et al.,* 2003). Various studies have been conducted to study effect of air plasma on seed germination ability,

plant height and root height and the results gave a positive correlation between seed germination rate and plant height without posing any adverse effects to nutritive properties of crops (Filatova *et al.,* 2013).

wheat varieties were taken for comparison on the basis of their antioxidant activity and total phenolic content. Different extraction methods were followed for the preparation of antioxidant extract from wheat. Solvent system used for extraction were 70% methanol (v/v), 50% acetone(v/v) and 70% ethanol(v/v). The results showed that different approach for extraction purpose leads to difference in antioxidant activity and total phenolic content of wheat. The comparison was done between the whole wheat and bran portion of wheat which showed that bran fraction posses more phenolic content. Conclusion of this paper was that wheat consumption is among the healthier meal (Safaa S. Abozed *et al.,* 2014).

Atmospheric cold plasma is a tool known for its efficiency for microbial and fungal decontamination on various food materials. Further the implementation of this method was done to ameliorate the quality maintenance of various fruits. The fruit they have choosen was freshly cut kiwi fruit, therefore the sample was under the exposure of plasma at two different exposure time 10 and 20 min and then stored under controlled conditions for four days. Texture, visual quality, chlorophyll, polyhenols and caretonoids were monitored. Antioxidant activity were also studied. Hence the results showed that plasma treatement have effected the quality of fruit by enhancing color retention and minimized the darken area formed during storage. Therefore, no significant difference in the antioxidant activity was observed between plasma treated and untreated kiwi fruit (Ileana Ramazzina *et al.,* 2015).

The effect of milling procedure on different varieties of wheat were studied to evaluate that is there any difference between their antioxidant activities. It was observed by this experiment that total pehnolic content and antioxidant activity is higher in whole wheat flour when compared with refined flour. Further the formation of steamed bread and noodles from the flour by undergoing different processes the reduction in the total flavonoid content, phenolic content and antioxidant activity is observed (Yao guang Li *et al.,* 2015).

Low pressure cold plasma an innovative technology rapidly being used for surface modifications purposes in various fields. However, this technology is of considerable interest to the scientists and various food industries due to its broad area of application. This technology is being used to escalate the surface energy of materials so that the water uptake capacity of material can be enhanced. Low pressure cold plasma has been employed to study various physicochemical and cooking quality of Basmati rice. As the surface modification, etching and increased hydrophobic interactions of rice grains can be useful in reducing the cooking time of rice leading to minimum resource utilization. Increase in the whiteness index of the basmati rice was observed with an increase in power of plasma and duration. (Thirumdas *et al.,* 2015).

Plasma treatment is able to modify the textural properties which result in reduction of hardness and stickiness of rice. (Denis Butscher *et al.,* 2016)

3. Aims and Objectives

The aim of this project "To Study the Effect of Plasma Treatment on Fungal Decontamination in Wheat and Biochemical Parameters Thereof "

Main Objectives:

1. To study the effect of plasma treatment on fungal decontamination in Wheat (*Triticum aestivum)*
2. To analyze the effect of plasma treatment on physical and biochemical parameters of wheat (*Triticum aestivum)*

4. Materials and Methods

4.1 Sample collection

The *Triticum aestivum* (Wheat) variety HD3086 was procured from Punjab Agriculture University, Ludhiana. The collected seed samples were kept in pouches and preserved at 4°C until use.

4.2 Procurement of Test pathogen

Aspergillus flavus (MTCC 871) was obtained in lyophilized form from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India

4.3 Activation of pathogenic culture

Activation of the lyophilized cultures was done by adding 200µl of saline in the lyophilized ampule of culture followed by spreading 20µl of this culture suspension on to Czapek Dox Agar plates under aseptic environment. Further, plates were incubated at 26 ± 2˚C for 7-10 days. The cultures thus obtained were maintained as pure culture on Czapek Dox Agar slants supplemented with 10% (v/v) glycerol.

4.4 Preparation of Spore suspension

To the actively growing pure culture of *A. flavus* (MTCC 871), 5 ml of sterile saline solution supplemented with 0.1% (v/v) Tween 80 was added and aseptically spores suspension was collected in a sterile centrifuge tube. The spore suspension was centrifuged at 10,000 rpm for 10 min to obtain spore pellet. After centrifugation, the supernatant was discarded and spore pellet was re-suspended in physiological saline. The optical density of the spore suspension was measured at 530 nm and further diluted to obtain $1x10^6$ spores/ml in physiological saline solution containing 0.1% (v/v) Tween 80.

4.5 Preparation of wheat grains for plasma treatment

4.5.1 Surface Sterilization

Seeds of *Triticum aestivum* (Wheat) were surface sterilized by soaking in 0.5% sodium hypochlorite for 2 min followed by rinsing with double distilled water three times. The seeds were then allowed to dry over filter paper under aseptic conditions. The dried seeds were further subjected for *A. flavus* infection.

4.5.2 Inoculation of Spore Suspension on Wheat Grains

Dried Wheat grains (3g) were sprayed with 600 µl of fungal suspension, inverted few times and incubated at 30˚C for 15-16h. The initial concentration of spores was

confirmed by plating the infected grains onto Czapek Dox Agar plates and CFU/g was determined.

4.6 Plasma Treatment

Plasma treatment was carried out for the decontamination of the wheat sample infected with *A.flavus*. After the incubation is over, the *Aspergillus* infected wheat grains were placed inside the low pressure Plasma reactor (PICO model, Diener electronic, Germany). The plasma reactor consisted of rotary drum containing stainless steel chamber, vacuum pump and matching network. The pressure and temperature of plasma rector was adjusted to 0.5 mbar, 28˚C respectively (Seluck *et al.,* 2008). The pressure and temperature of reactor was maintained by using the pressure gauges and thermocouples respectively. The plasma reactor internal chamber was supplied with SF_6 gas for 10 min and flow of gas was regulated by mass flow controllers.

4.7 Cell Viability Determination

To determine the number of viable cells present after the plasma treatment, different dilutions ranging from 10^{-1} to 10^{-5} cells/ml of the plasma treated and untreated sample were prepared. 100µl of each dilution was spread on to Czapek Dox Agar plates and incubated for 72 hours at 30˚C. After incubation, numbers of colonies were counted with the help of colony counter and the results were expressed as log colony forming units per gram (log CFU/g).

4.8 Effect on cooking properties of wheat after plasma treatment

4.8.1 Cooking Time of Wheat

2g of plasma treated and untreated wheat sample in the form of porridge was crushed in a pestle and mortar and the contents were transferred into a test tube followed by addition of 20 ml of distilled water. The test tubes were kept at boiling water bath and checked for

optimal cooking by taking out samples at different time intervals and by pressing them between glass plates. (Chen *et al.*, 2012).

4.8.2 Water Uptake of Wheat

2g of plasma treated and untreated wheat sample in the form of porridge was cooked in a test tube for an optimized cooking time in a boiling water bath. Thereafter, the cooked sample was placed on a filter paper to drain off excess water and moisture from sample surface. Subsequently, the weight of the sample was noted to estimate the water uptake capacity (Singh *et al.*, 2005).

4.9 Proximate analysis of Plasma treated wheat *(Triticum aestivum*) grains

4.9.1 Estimation of Moisture Content

To determine the effect of the plasma treatment on moisture content of wheat grains, two grams of treated and untreated wheat grains were placed in a pre-weighed dried crucible (W_1) and its weight was noted, designated as W_2. Subsequently, crucibles containing wheat grains were incubated at 130˚C for 1 hour and then allowed to cool inside a desiccator. The crucibles were again weighed and weight was designated as W_3. (AOAC, 2010). The % age moisture content of the sample was calculated by using following formula

$$\% \text{ Moisture content} = (W_2 - W_3 / W_2 - W_1) \text{ X } 100$$

Where, W_1= weight of empty dried crucible, W_2= weight of crucible containing wheat grains, W_3 = Final weight of crucible containing wheat grains after incubation.

4.9.2 Estimation of Ash Content

To estimate the ash content, two grams of plasma treated and untreated wheat grains were placed in a pre-weighed dried crucible (W_1). Crucible containing wheat grains were weighed and weight was designated as W_2. Further, these crucibles were placed in Muffle furnace at 600ºC for 6 hours. After 6 hours, crucibles were taken out and allowed to cool in the desiccator. The final weight of the crucibles were noted and designated as W_3 (AOAC,2010). The % age ash content of the sample was calculated by using following formula

$$\% \text{ Ash content} = (W_3 - W_1 / S) \times 100$$

Where W_1 = weight of empty dried crucible, W_3 = Final weight of crucible containing wheat grains after Muffle furnace treatment.

4.9.3 Estimation of Fat Content

The fat content of plasma treated and untreated samples were estimated as described by AOAC, 2010. Briefly, 2 g of treated and untreated wheat sample was grinded and kept in thimble with a top glass wool. To the pre-weighed (W_1) round bottom flask, 5-6 glass beads were added followed by addition of 300 ml of petroleum ether into the flask. Simultaneously, thimbles containing the samples were placed in glass jacket kept onto pre-labeled extraction flasks. A condenser is fitted over the glass jacket for reflux. Cold tap water was percolated through the condenser for condensation purpose which promote in refluxing the ether in the thimble back, the unit was turned on and was allowed to reflux for 6 hours. After switching off the system, the remaining solvent was extracted from the sleeve in the flask. Further the flask was incubated at 130˚C in oven for 3.30 h, allowing the ether to evaporate out leaving behind the extracted oil at the bottom of the flask. Flasks were placed in the desiccators to cool down and the weight was taken as W_2. The percentage fat content was calculated by using the formula.

$$\% \text{ Fat content} = (W_2 - W_1)/ S \times 100$$

Where, W_1 = weight of empty round bottom flask, W_2 = Final weight of the flask after experiment, S = weight of the sample

4.9.4 Estimation of Carbohydrate Content

The total carbohydrate content of plasma treated and untreated sample was estimated by using Anthrone method (Yemm and Willis 1954). This method is used to detect both reducing and non- reducing sugars under acidic conditions with the formation of blue green color. Different concentrations of glucose ranging from 20 ppm to 100 ppm was prepared. Briefly, 4ml of anthrone reagent was added to 1ml of each dilution and plasma treated and untreated sample extract. Further, the tubes were vortexed and kept in boiling water bath for 10 minutes followed by cooling at room temperature. The absorbance of

the solution was measured at 620nm. Test tube containing only anthrone reagent was used as blank. The test was performed in triplicates.

4.9.5 Estimation of Protein Content

2 g of fine grinded plasma treated and untreated wheat samples were added in a round bottom long neck conical flask followed by addition of 15 ml of concentrated sulfuric acid (H_2SO_4). Subsequently, Potassium sulfate and copper sulfate was added in ratio 8:1 and flask was kept for 2 hours on the digestion unit for acid digestion. After acid digestion, sample was cooled down to room temperature. Further the sample was diluted to 140 ml using DI Millipore water followed by addition of 50% concentrated sodium hydroxide (NaOH) solution with a few glass beads. The solution was distilled using Kjeldhal distillation unit (Model unit B- 45). The Liberated ammonia is entrapped in 4% boric acid solution with methyl red indicators. Finally, the solution was titrated against 0.01 N HCl. Blank reaction was set up without sample (AOAC, 2010).

% of Nitrogen = {(S-B) *1.4007* 0.1}/ Weight of sample

% of Protein content = 5.7 X N (%)

Where, S = sample burette reading from titration, B = blank burette reading from titration

4.10 Fourier Transform Infrared (FTIR) Spectroscopy

Plasma treated and untreated wheat grains were grounded to fine powder in a mortar and pestle and then sample is added 1% of KBr. The contents were mixed properly and a very fine layer of this mixture was placed under a pressure of 110 psi in hydraulic pump for 2 min. After the release of pressure, the pellet was taken out and kept in desiccator for 5-10min. The prepared KBr pellets of samples were placed in FTIR Varian 600 IR series apparatus to detect any changes in absorption spectrum of plasma treated and untreated sample.

4.11 Effect on Protein Profiling

4.11.1 Protein Extraction and Estimation

For Protein extraction, 5g of plasma treated and untreated wheat grains were crushed in a mortar and pestle using ice-cold 50 mM Tris-HCl buffer, pH=7.5 and the contents were

transferred into micro-centrifuge tubes. Centrifugation was done at 10,000 rpm for 10 minutes at 4ºC. The pellet was discarded and supernatant (crude extract) was further subjected to Bradford's Assay to estimate the protein content. A standard curve of BSA was prepared against which the values of the unknown protein samples were plotted. Different concentrations of BSA (Stock- 1mg/ml) were prepared and protein content was estimated using Bradford's method in a 96 well titer plate. To each well, 10µl of each BSA concentration and protein samples were dispensed followed by addition of 90µl of Bradford's Reagent. The plate was incubated at room temperature for 15 mins and absorbance was measured at 595 nm using Biotek Powerwave 340 Microplate Reader. All the tests were performed in triplicates and values were expressed as Mean ± SD.

4.11.2 SDS-PAGE

For SDS- PAGE, 10% resolving and 5% stacking gel was prepared according to the composition as described in Table 1 and gel was allowed to solidify. Simultaneously, the sample was prepared by adding 20µl of crude extract of plasma treated and untreated samples with 10µl of 5X loading dye and incubated at boiling water bath for 3 min. After solidification of gel, the samples were loaded onto wells. A molecular weight marker of 29KDa to 205KDa was also loaded as standard marker. Gel was allowed to run at 80V for 3 hours in 1X electrophoresis buffer until the tracking dye reaches at the bottom. After the gel electrophoresis was done, the gel was stained with staining solution (Methanol - 20%, Glacial Acetic Acid -10 %, Coomassie Blue R-250 - 0.15 %) for 2-3 h and further subjected to de-staining solution ((Methanol - 20%, Glacial Acetic Acid -10 %) for the visualization of protein bands.

Ingredients	Separating gel (10%)	Stacking gel (5%)
Autoclaved Double distilled water	2.20 ml	2.50 ml
30% Acrylamide: bis-acrylamide	2.20 ml	980 µl
1.5M Tris HCl (pH 8.8)	1.25 ml	----

0.5M Tris HCl (pH 6.8)	----	1.25 ml
10% SDS	50 µl	70 µl
10% Ammonium persulfate	50 µl	70 µl
TEMED	06 µl	07 µl

Table 1 Composition of Separating and stacking gel of SDS-PAGE

4.12 Determination of Antioxidant potential by FRAP Assay

FRAP assay is mostly used for the evaluation of the presence of antioxidant components in dietary poly-phenols. The extraction of wheat sample was done by (Moore *et al.*, 2006). Firstly, 2g of crushed plasma treated and untreated wheat grains were extracted thrice with 20 ml of 50 % Acetone by vortexing for 20 min. Centrifugation was done at 3,000 rpm for 10 minutes to remove cell debris. Subsequently, 3 ml of FRAP reagent (10ml of 300mM acetate buffer (pH 3.6), 1ml of 10mM of TPTZ in 40mM HCl, 1ml of 20mM $FeCl_3.6H_2O$) was added to 100 µl of each crude extract. 50% acetone was used as a control. The sample was incubated at 37˚C for 15 min in dark. Then the absorbance was measured at 593nm at UV-VIS spectrophotometer (Varian co.) (Benzie and Strain 1996).

4.13 Estimation of Total Phenolic Content

Determination of total phenolic content was done as per the method of Folin–Ciocalteu reagent (Yu *et al.*, 2003). To 0.5ml of crude extract of plasma treated and untreated wheat samples, 2.5 mL of Folin-Ciocalteu reagent (10 %) was added, followed by addition of 2.5 ml of 7.5% $NaHCO_3$. All samples were incubated at 45^o C for 45 minutes. After incubation, absorbance was measured at 765 nm. Gallic acid was taken as standard. A standard curve of gallic acid was prepared by using different concentrations (10ppm – 200ppm). The phenolic content of wheat samples were calculated by plotting the values in gallic acid standard curve.

4.14 Estimation of Total Flavanoid Content

The total flavanoid content of plasma treated and untreated wheat grains were performed using aluminium chloride colorimetric assay with slight modifications (Zhishen *et al.*, 1999). The method involves dilution of 1 ml of sample extract with 4 ml of double distilled water followed by addition of 300 µl of 5% $NaNO_2$. The mixture was incubated

at 37˚C for 5 min and then 300 µl of 10% $AlCl_3$ and 2 ml of 1M NaOH was added into it. Then make up the volume to 10 ml with distilled water, and measure the absorbance at 510 nm. Catechin was used as control and a standard curve of catechin was prepared by using different concentrations ranging from 10ppm to 250ppm. All the tests were performed in triplicates and values were expressed as Mean ± SD.

5. Results and Discussion

5.1 Plasma Treatment

Plasmas are being used in range of commercial processes viz. material processing, anticorrosion coating, Hazardous waste treatment, microelectronic technology. Despite of wide application in aforesaid areas, plasma treatment has been recently employed in sterilization and decontamination of food surfaces. The basic principle behind the inactivation pattern of microorganisms by plasma treatment is the destruction of the cell wall of bacteria or fungus by the extreme application of electrons or ion bombardments (Moisan *et al.,* 2001). In present study, *Aspergillus flavus* infected wheat grains were exposed to plasma stemming from an electrical stimulation in presence of SF_6 gas. The SF_6 gas posses no biocidal effect on surface contamination of wheat grains until they were activated by electrical stimuli. The wheat grains were subjected to various exposure durations ranging from 2- 20 min.

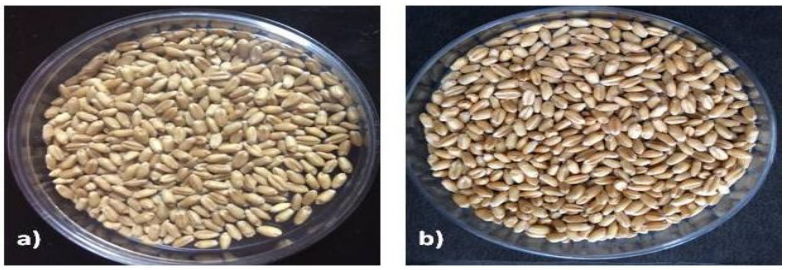

Figure 2 showing Wheat grains a) Untreated b) Plasma treated

5.2 Disinfection effect of Plasma Treatment on *Aspergillus flavus*

The decontamination of *Aspergillus flavus* from the wheat sample was performed by using SF_6 gas and exposing then for varying durations ranging from 2 min to 20 min (Figure 3).

Duration	**CFU/g**	**Log_{10} CFU/g**
0 minute	7.5×10^6	6.87
2 minute	3.4×10^5	5.53
5 minute	0.8×10^4	3.90
10 minute	0	0

Table 2 CFU/g and Log_{10} CFU/g of fungal spores on plasma treated and untreated wheat

The total number of fungal spores before plasma treatment was 7.5 X 10^6 CFU/g. However, after plasma treatment of 5 min, the total number of fungal spores was reduced to 0.8 X 10^4 CFU/g (Table 2).

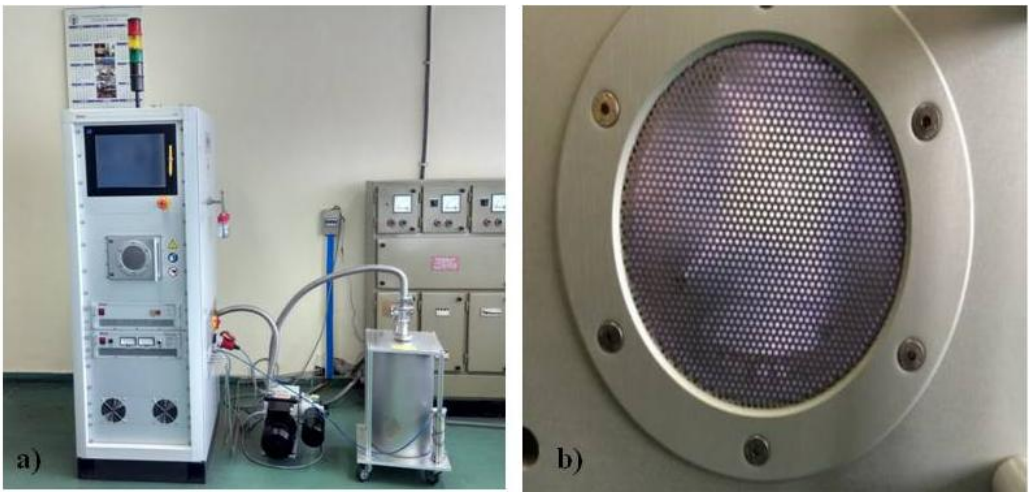

Figure 3 showing (a) low Pressure Plasma reactor PICO (b) plasma chamber

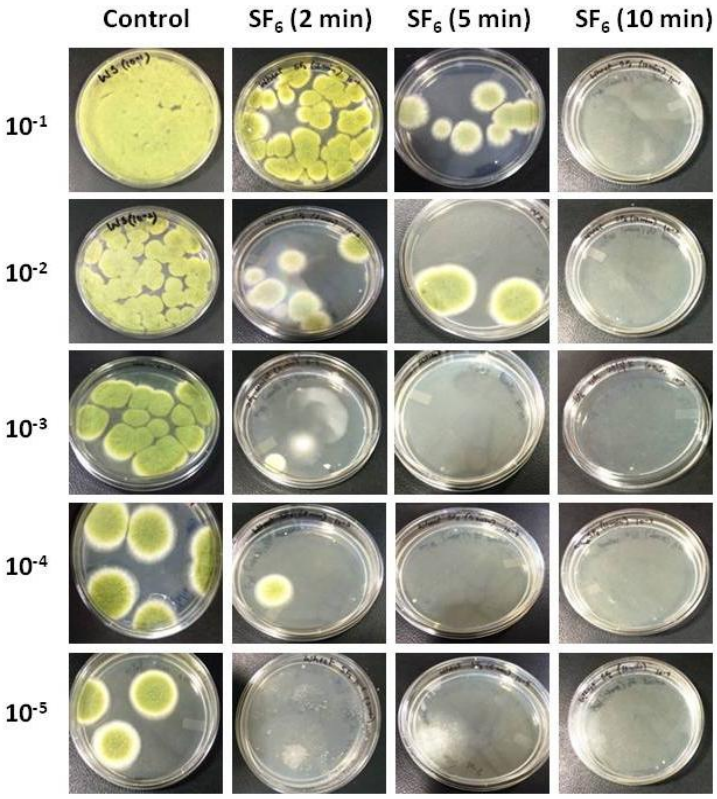

Figure 4 Colonies of different dilutions of untreated and plasma treated sample for different durations

Further, 1- log inhibition of fungal spores was observed after 2 min of plasma treatment which was escalated to 2-log inhibition after 5 min of plasma treatment. Further, infected wheat grains samples were subjected for plasma treatment for post 10 min duration but on further extending the exposure time, the reduction effect remains stationary (Figure 4). The optimal duration of SF_6 plasma treatment was found to be 10 min with complete inhibition of microbial population.

5.3 Effect of Plasma Treatment on Cooking Properties

5.3.1 Cooking time

The cooking time for plasma treated and untreated wheat sample showed significant difference ($p < 0.05$). The cooking time of plasma treated sample was reduced to 6.3 min from 7.4 min. The probable reason of reduced cooking time can be easy penetration of water into the plasma treated wheat surface (Figure 5). The reduction of cooking time is of prime importance as it helps in prevention of resources used for cooking process.

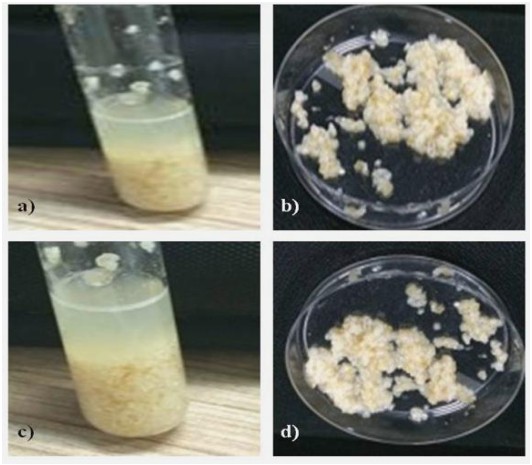

Figure 5 showing cooked sample (a-b) plasma treated (c-d) untreated sample

5.3.2 Water Uptake Capacity

As plasma treatment is defined as surface phenomena, modifications in the surface were observed. The plasma treated sample looks shiner as compared to untreated sample, because plasma results in surface modification such as etching. Water uptake capacity of plasma treated sample was increased from untreated sample (Table 3). The increase in water uptake ratio can be correlated with reduction in cooking time of plasma treated sample also reported by (Mohapatra and Bal 2006).

Sample	**Initial weight (A)**	**Final weight (B)**	**Water uptake (B/A)**
Plasma treated sample	2.0041	8.5679	4.275
Untreated sample	2.0057	8.2188	4.097

Table 3 showing water uptake capacity (in grams) of plasma treated and untreated wheat sample

5.4 Proximate Analysis of Wheat *(Triticum aestivum*)

5.4.1 Moisture Content Estimation

The presence of moisture content in the bulk grains provides an appropriate environment for fungal growth, the control of moisture content is very necessary in order to prevent post harvest loss in stored grains. The initial moisture content of untreated wheat grains was found to be 9.86%. There was a considerable reduction in moisture content of plasma treated wheat grains. By the application of plasma treatment, moisture content of grains reduced from 9.86% to 8.68% (Table 4). The reason for moisture value reduction may be formation of oxygen radicals by the decomposition of water molecules (Zou *et al.*, 2004).

Figure 6 wheat sample present in dessicator

Sample	W1	W2	W3	Moisture (%)
Plasma treated sample	19.5082	21.5084	21.3347	8.68
Untreated sample	18.937	20.9450	20.747	9.86

Table 4 showing percentage moisture content of plasma treated and untreated wheat sample

5.4.2 Ash Content Estimation

Ash content is defined as the mineral content of flour. Determination of ash content in food sample is done by burning particular quantity of grains (Figure 7). Large amount of minerals in the wheat kernel are present in the bran. So the presence of ash content is directly proportional to the bran particles in the flour. The initial ash content of untreated wheat grains was found to be 1.5%. There was very slight increase (+ 0.1%) in ash content of plasma treated wheat grains (Table 5).

Figure 7 Ash content present in plasma treated and untreated wheat sample

Sample	W1	W2	W3	% Ash
Plasma treated wheat	19.020	2.015	19.052	1.6
Untreated wheat	18.811	2.027	18.841	1.5

Table 5 showing percentage ash content of plasma treated and untreated wheat sample

5.4.3 Fat content estimation

The initial fat content of untreated sample was found to be 0.4%. However, fat content was found to be increase slightly in plasma treated sample in comparison to untreated sample (Table 6). Variation in the fat content value in treated and untreated sample may be due to the reduction in moisture content of sample or oxidation of the free radicals present. These differences varies depending upon the parameters decided for plasma treatment application (Lii *et al.*, 2002a, 2002b)

Sample	Initial weight (W_1)	Final weight (W_2)	% Fat $(W_2-W_1)/S * 100$
Plasma treated wheat	76.483	76.582	0.49
Untreated wheat	82.886	82.894	0.4

Table 6 showing percentage fat content of plasma treated and untreated sample

5.4.4 Carbohydrate Content Estimation

The initial carbohydrate content of untreated wheat grains was 3.30µg/ml which was calculated from standard curve of Glucose (Figure 8). Further, plasma treated samples showed slight increase (+0.7µg/ml) in carbohydrate content viz. 4.01µg/ml. During post harvest storage, there is high probability of degradation of carbohydrate and proteins present in grains because of the presence of the mycotoxins released by pathogenic fungi. The application of plasma treatment helps in maintaining nutrient value of grains.

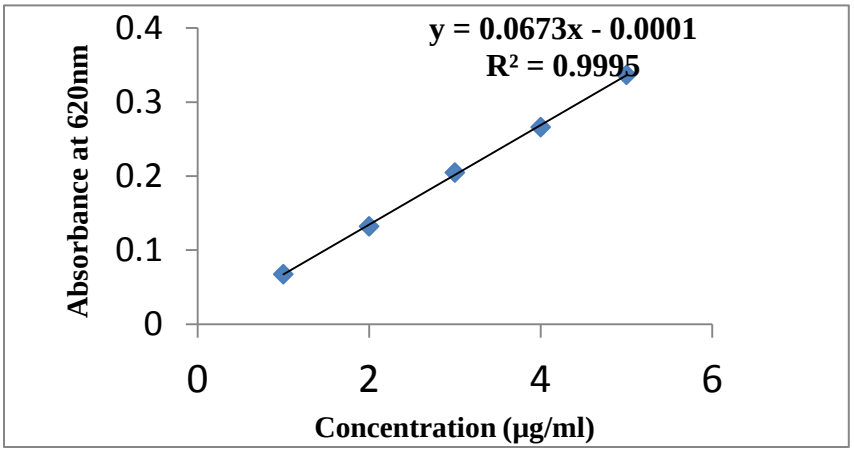

Figure 8 Standard curve of Glucose

5.4.5 Protein Content Estimation

The Protein content of plasma treated sample exhibited slight increase in protein content in comparison to untreated sample. The protein content of plasma treated sample was increased by 1% i.e. 11.390% in comparison to 10.143% of untreated sample (Table 7). Increase in the protein content can be correlated with slight breakage of the proteins present on the surface and other proteinaceous matter because of the oxygen which plays a major role in degradation reaction (Deng *et al.*, 2007)

Sample	**Initial value (B)**	**Final value (S)**	**% Nitrogen ((S-B)* 1.4007* 0.1)/2**	**% Protein**
Plasma treated sample	0	28.3	1.981	11.390
Untreated sample	0	25.2	1.764	10.143

Table 7 showing percentage protein content of plasma treated and untreated sample

5.5 FTIR Analysis

FTIR is an efficient method which can be used to analyze variability in functional groups of various phytochemicals of plasma treated wheat sample. FTIR spectra of both plasma treated and untreated wheat sample did not impart any variability (Figure 9).

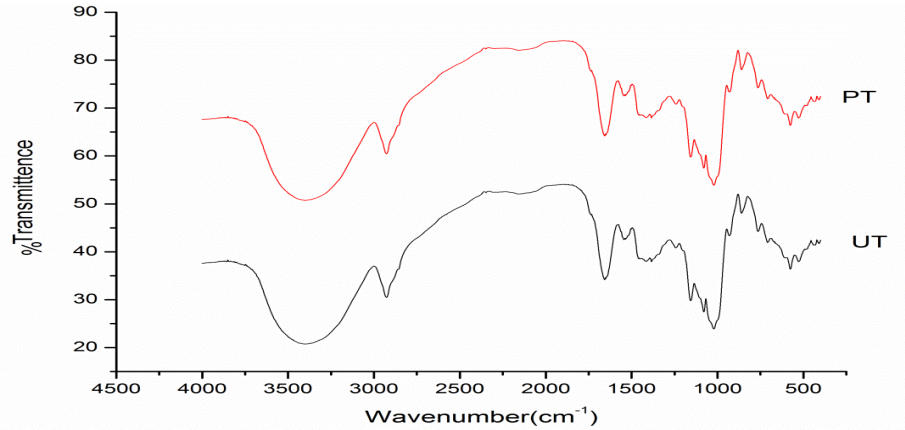

Figure 9 showing FTIR spectrum of plasma treated and untreated wheat grains

5.6 Effect on Protein Profiling

There was slight difference in the protein concentration of plasma treated and untreated sample. The protein concentration of plasma treated and untreated sample was found to be 0.791µg/ml and 0.748µg/ml as calculated by plotting the absorbance values in the equation, y = 0.745x - 0.0002 of standard curve of BSA (Figure 10).

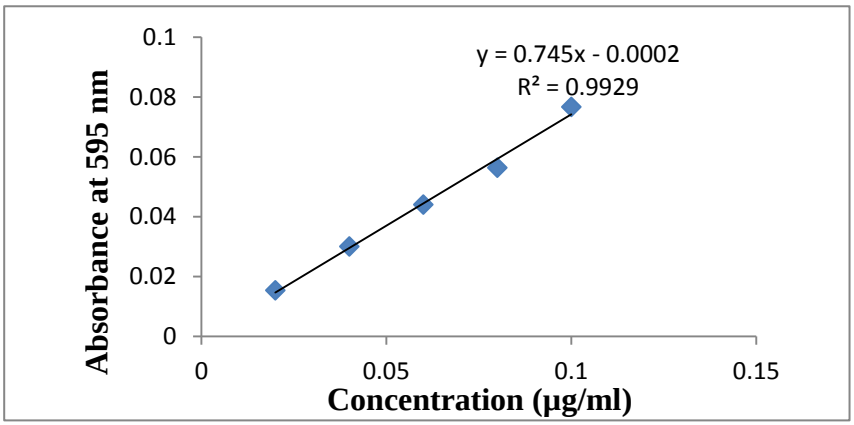

Figure 10 showing Standard curve of Bovine Serum Albumin (BSA)

5.6.1 SDS –PAGE

The variation in the seed storage proteins profiling was checked by running SDS-PAGE (Figure 11). The bands of protein observed in plasma treated (lane 1 and 2) were same as that of untreated sample protein (lane 3 and 4). There was no difference in molecular weight of protein bands in treated as well as untreated samples thereby confirming that plasma treatment with SF_6 gas did not exert any negative effect on the nutritive value of wheat grains.

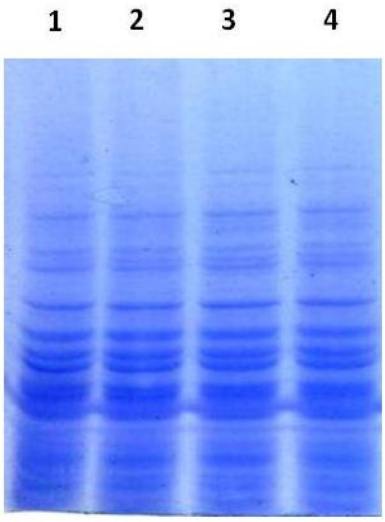

Figure 11 showing SDS-PAGE gel of plasma treated (lane 1, 2) and untreated sample (lane 3,4)

5.7 Antioxidant Activity

5.7.1 FRAP Assay

FRAP analysis estimates the ability of plasma to reduce ferric, it helps in detection of antioxidant potential. The antioxidant activity of plasma treated sample was found to be increased by two fold in comparison to untreated sample. The antioxidant activity of plasma treated and untreated sample was found to be 0.0443mg/g and 0.0185mg/g respectively as calculated from standard curve of ascorbic acid (Figure 12). The probable reason for higher antioxidant activity can be correlated with increased ability to reduce ferric ions as compared to untreated sample.

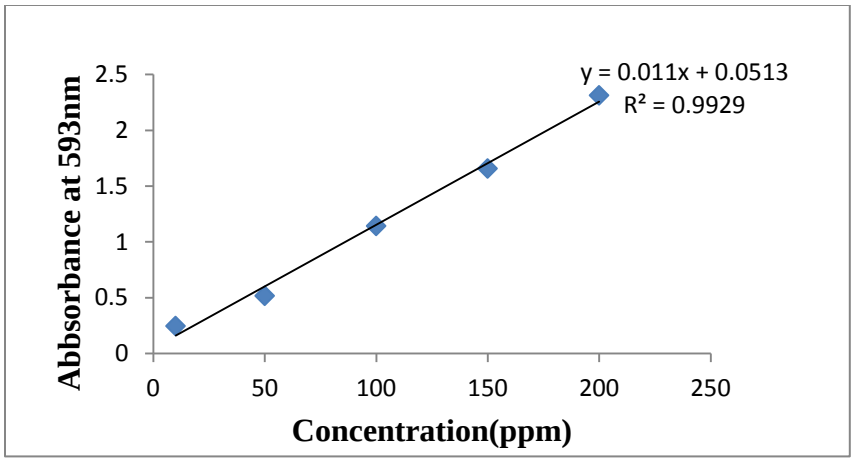

Figure 12 showing standard curve of Ascorbic acid

5.7.2 Total Phenolic Content

The total phenolic content (TPC) of plasma treated and untreated sample was calculated by preparing a standard curve of gallic acid (Figure 13). The TPC was calculated by putting absorbance values in equation, y = 0.0093x-0.2175. The TPC of plasma untreated sample was found to be 0.1368 mg/g. However, plasma treated samples showed a slight increase in total phenolic content i.e. 0.1467 mg/g in comparison to untreated samples.

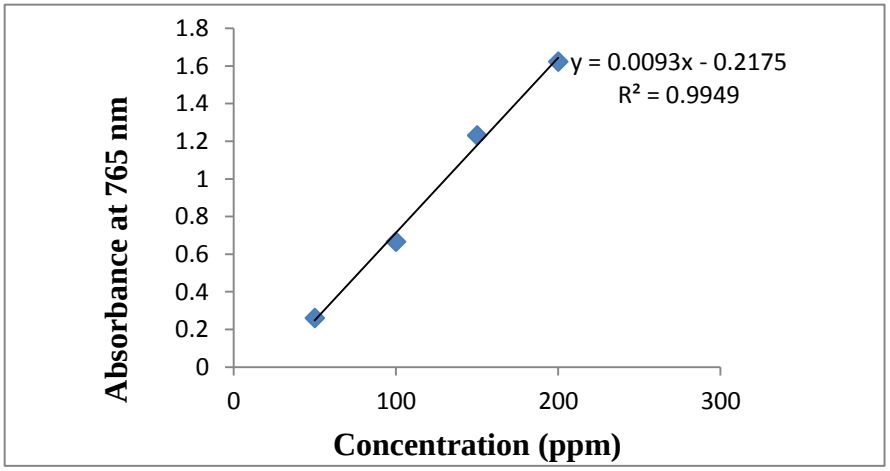

Figure 13 showing Standard curve of Gallic Acid

5.7.3 Total Flavonoid Content

The total flavanoid content (TFC) corresponds to polyphenol content present in the wheat bran. The flavanoid content of plasma treated and untreated wheat sample was calculated by putting the absorbance values in equation, y = 0.0033x + 0.0186, of standard curve of catechin (Figure 14). The flavanoid content of untreated wheat sample was found to be 0.0167 mg/g which was found to increase by one fold in plasma treated wheat sample. The total flavanoid content of plasma treated sample was found to be 0.0216mg/g.

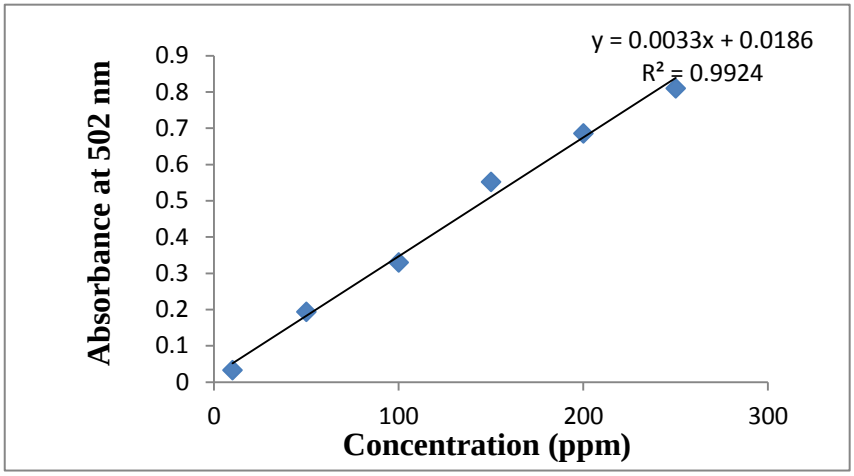

Figure 14 showing standard curve of Catechin

6. Conclusion

The current study showed that plasma treatment can be considered as most efficient method for fungal decontamination as well as surface modification of seeds and grains. Low pressure plasma system did not have any deleterious effect on the seeds and grains. SF_6 gas showed great efficacy in *A. flavus* spores reduction present on the surface of wheat grains. The minimum duration required for the grains to undergo plasma treatment for complete destruction of fungal spores was found to be 10min. Reduction in the moisture content and cooking time was observed by this application. Protein profiling of plasma treated and untreated sample was done by SDS-PAGE, but no difference in the bands were observed. Slight increase in antioxidant activity of grains was found. Thus plasma treatment can be used to enhance biochemical parameters of seeds and grains with respect to fungal decontamination.

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