



EFFECT OF YEAST FERMENTATION PROCESS ON

ANTI-

NUTRIENTS AND VALUE ADDITION TO

ALMONDS

A PROJECT REPORT

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BONAFIDE CERTIFICATE

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ABSTRACT

The almond belongs to the same group of plants as the rose, plum, cherry and peach, being a member of the tribe *prunae* of the natural order *Rosaceae*. The almond is grown for its edible nuts which are either consumed as such or used in confections and puddings. Almond is chiefly valued because of its virtue of preventing intoxication, its high fat, protein, fibre, vitamin and mineral contents. Almonds reduce low-density lipoprotein (LDL) cholesterol, while preserving the beneficial high-density lipoprotein (HDL) cholesterol. Anti-nutrients are natural or synthetic compounds that interfere with the absorption of nutrients. Anti-nutrients like phytin, tannin and oleic acid are found in almonds. Phytic acid, known as inositol hexakisphosphate (IP₆), or phytate when in salt form is the principal storage form of phosphorus in many plant tissues, especially bran and seeds. Phytic acid reduces the absorption of valuable minerals such as calcium, iron, magnesium, and zinc by binding the minerals and forming an insoluble salt in the human body. Thus by reducing the anti nutrient content in almonds, the valuable minerals can be absorbed better by the human body. One of the methods to reduce the phytic acid content is fermentation process. Yeast was isolated from fruits like papaya and chikoo and they were used to ferment the almonds thereby reducing their anti nutrient content.

Keywords: Lipoprotein, cholesterol, anti-nutrient, tannin, oleic acid, fermentation.

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LIST OF ABBREVIATIONS

LDL	Low Density Lipoprotein
HDL	High Density Lipoprotein
IP ₆	Inositol Hexaphosphate
YPD	Yeast Peptone Dextrose
Spp.	Species
Fe(NO ₃) ₃	Ferric Nitrate
KSCN	Potassium thiocyanate
rpm	Revolutions per minute
nm	Nanometre
ml	Millilitre
μl	Microlitre
μg	Microgram
CFU	Colony Forming Units
mins	Minutes
TCA	Trichloroacetic acid
NaOH	Sodium Hydroxide

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especially rich in mono-unsaturated fatty acids like oleic and palmitoleic acids that help to lower LDL or "bad cholesterol" and increase HDL or "good cholesterol." Research studies suggest that Mediterranean diet, which is rich in monounsaturated fatty acids helps to prevent coronary artery disease and strokes by favouring healthy blood lipid profile.

The nuts are an excellent source of vitamin E; contain about 25 g per 100 g. Vitamin E is a powerful lipid soluble antioxidant, required for maintaining the integrity of cell membrane of mucus membranes and skin by protecting it from harmful oxygen free radicals. Almonds are free in gluten and therefore, are a popular ingredient in the preparation of gluten free food formulas. These nuts are packed with many important B-complex groups of vitamins such as riboflavin, niacin, thiamin, pantothenic acid, vitamin B-6, and folates. These vitamins functions as co-factors for enzymes during cellular substrate metabolism. They are also rich source of minerals like manganese, potassium, calcium, iron, magnesium, zinc, and selenium.

The sweet almond oil is obtained from the nuts is an excellent emollient that helps to keep skin well protected from dryness. It is also used in cooking, and as "carrier or base oil" in traditional medicines in aromatherapy, in pharmaceutical and cosmetic industry. Almonds yield nearly half their weight in a bland fixed oil, which is employed medicinally for allaying acid juice, softening and relaxing solids and in bronchial diseases, in tickling coughs, hoarseness, costiveness, nephritic pains. Also, as they contain practically no starch and being rich in protein, they are often made into flour for cakes and biscuits for diabetic patients.

1.3 Anti-nutrients

Anti-nutrients are natural or synthetic compounds that interfere with the absorption of nutrients. One common example is phytic acid, which forms insoluble complexes with calcium, zinc, iron and copper. These compounds chelate metals such as iron and zinc and reduce the absorption of these nutrients, but they also inhibit digestive enzymes and may also precipitate proteins. Many traditional methods of food preparation such as fermentation, cooking, and malting increase the nutritive quality of plant foods through reducing certain anti-nutrients such as phytic acid, polyphenols, and oxalic acid. In almonds the concentration of phytic acid is higher. Phytates are just one of many anti-nutrients in grains, nuts, tubers, seeds and beans. Others include oxalates, tannins, trypsin inhibitors, enzyme inhibitors,

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CHAPTER 1 INTRODUCTION

1.1 General

Almond is a nut belonging to the order Rosales, family Rosaceae, genus *Prunus* and subgenus *Amygdalus*. Almond is one of the most nutritious nuts. They contain dietary fibre, vitamins, minerals, monounsaturated fatty acids along with anti-nutrients. Almond is a seed of drupe i.e., a fruit in which an outer fleshy part surrounds a shell of hardened endocarp with a seed inside. The almonds are native to the northern Indian sub continent, and the belt lining Syria, Israel and Turkey. They are also found along the shore of the Mediterranean into northern Africa and southern Europe, as well as in parts of California. Almonds are of two types, sweet and bitter. The kernel of sweet almonds is used for extracting fixed oils and emulsions. It is also used in alternative medicine commonly as an oil in aromatherapy. The bitter almonds are comparatively broader and shorter than the sweet one. It contains an enzyme which in the presence of water yields glucose, cyanide and the essential oil.

1.2 Nutritional characteristics of almond

The study of nutritional characteristics of Indian almond (*Prunus amygdalus*) showed the approximate composition to be: 97.70% dry matter, 11.52±1.10% crude protein, 6.76±0.72% ash, 5.09±0.84% crude fibre, and 21.76±1.20% fat and 54.87±2.80% carbohydrate. The anti-nutrient composition was found to be 0.25% phytin, 0.07% phytin phosphorus, 0.04% cyanide, 1.82% Tannin. Its fatty acid and mineral contents were 0.65% oleic acid, 0.19% phosphorus, 0.17% potassium, 0.25% magnesium, 245.65 ppm sodium, 845.45 ppm calcium, 92.12 ppm zinc, 70.62 ppm iron and 9.21 ppm copper.

1.2.1 Health benefits of almonds

Almond nuts are rich in dietary fibre, vitamins, and minerals and are packed with numerous health promoting phyto-chemicals; the kind of well-balanced food to ensure protection against diseases and cancer. These nuts are a rich source of energy and nutrition. They are

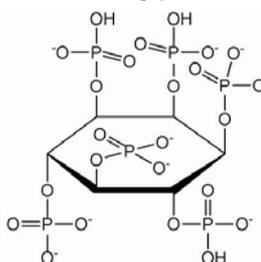
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lectins (hemagglutinins), protease inhibitors, gluten, alpha-amylase inhibitors and alkylresorcinols. For the plant, they perform many functions, such as protection against insects, mold and fungus. For humans, these can lead to impaired digestion, strain on the pancreas, and depletion of body resources.

1.4 Phytic acid

Phytic acid (known as inositol hexakisphosphate (IP₆), or phytate when in salt form) is the principal storage form of phosphorus in many plant tissues, especially bran and seeds. Phytate is not digestible to humans or nonruminant animals, however, so it is not a source of either inositol or phosphate if eaten directly. Moreover, it chelates and thus makes unabsorbable certain important minor minerals such as zinc and iron, and to a lesser extent, also macro minerals such as calcium and magnesium. Catabolites of phytic acid are called lower inositol polyphosphates. Examples are inositol penta- (IP₅), tetra- (IP₄), and triphosphate (IP₃).

1.4.1 Structure of phytic acid



1.4.2 Phytic acid in animal and human nutrition

Phytic acid is the principal storage form of phosphorus in many plant tissues, especially the bran portion of grains and other seeds. It contains the mineral phosphorus tightly bound in a snowflake-like molecule. In humans and animals, the phosphorus is not readily bioavailable. In addition to blocking phosphorus availability, the "arms" of the phytic acid molecule readily bind with other minerals, such as calcium, magnesium, iron and zinc, making them

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unavailable as well. In this form, the compound is referred to as phytate. Phytic acid not only grabs on to or chelates important minerals, but also inhibits enzymes that we need to digest our food, including pepsin, needed for the breakdown of proteins in the stomach, and amylase, needed for the breakdown of starch into sugar. Trypsin, needed for protein digestion in the small intestine, is also inhibited by phytate. Phytates not only prevent our absorption of important nutrients, but also inhibit enzymes we need to properly digest our food, including pepsin, amylase and trypsin.

Phytase is the enzyme that neutralizes phytic acid and liberates the phosphorus. This enzyme co-exists in plant foods that contain phytic acid. It is also produced in the digestive tract. Interestingly, animals with more than one stomach do not have problems with phytic acid-containing plants because large amounts of phytase are produced by rumen microorganisms.

1.4.3 Reduction of phytic acid

Foods containing phytic acid should be soaked, often in an acidic medium such as whey, buttermilk or yogurt, prior to consumption. This process, along with cooking in most cases, will activate phytase and greatly reduce the phytic acid. Sprouting activates phytase. Sprouting also releases vitamins and makes grains, beans and seeds more digestible. However, recent research shows that it is a pre-fermentation step, not a complete process for neutralizing phytic acid. For regular consumption, even sprouted grains should also be soaked and cooked. Fermentation is an excellent way to reduce phytic acid.

Those suffering with tooth decay issues should very strictly limit their phytic acid consumption, and children under six, pregnant women and those in ill health should consume as little phytic acid as possible.

1.5 Harmful effects of phytic acid

Phytic acid is considered as an anti-nutrient, it forms insoluble complexes with minerals such as zinc, calcium, magnesium and iron. During the pre-weaning period, the diet of an infant is based upon cereal flours as well as upon baby milk formulas. Therefore, the negative properties of phytates, particularly on the bioavailability of minerals, may have an effect on health during this first period.

All kinds of cereal grains and other products, such as roots (cassava) and seeds (peanuts, soy) are used to prepare the cereals for children. The infant, after 4 months of age, is at risk of developing an iron deficit as a result of a decrease of his organic deposits and the increase of his needs, determined by the higher growth velocity. This shortage may also be influenced by the presence of phytates in these cereal derivatives. Cereal porridges are common complementary foods during the weaning period and often provide much of the dietary iron intake because the iron contribution from human milk is low. Both cereal grains and legume seeds are rich in phytic acid. Iron absorption of native iron and fortification iron may be very low because of the high phytate content of cereal porridges; this was reported by Cook *et al.* (1997) and Hurrell *et al.* (1992). Also Navert *et al.* (1985) reported that we will absorb approximately 20% more zinc and 60% magnesium from our food when phytate is absent. In the same vein (Hurrell, 2003) reported the iron absorption rate in the wheat porridge increased to 12% when the phytic acid was removed.

Another set of experiments examined depression-fighting zinc and magnesium. Researchers provided two groups of people with bread. One group with bread with phytates and one with a control bread without phytates. Researchers then studied participants' mineral absorption via stool samples. Without phytic acid, participants absorbed about 30% of magnesium and zinc. With phytic acid, participants absorbed only 13% of their magnesium and 23% of their zinc (Egli *et al.*, 2002; Bohn *et al.*, 2004). The zinc-and iron-blocking effects of phytic acid can be just as serious as the calcium-blocking effects (Hallberg *et al.*, 1989) showed that a wheat roll containing 2 mg phytic acid inhibited zinc absorption by 18%; 25 mg phytic acid in the roll inhibited zinc absorption by 64% and 250 mg inhibited zinc absorption by 82%. Growing children run into severe problems in a phytate-rich diet, their bodies will suffer from the lack of calcium and phosphorus with poor bone growth, short stature, rickets, narrow jaws and tooth decay and for the lack of zinc and iron with anemia and mental retardation. Hurrell *et al.* (1998) showed that iron absorption in infants is inhibited by phytic acid in a way similar to iron absorption in adults.

1.6 Objectives

The Objectives of our work is:

- To isolate, purify and store the wild yeast spp. from various fruit sources.
- To determine the anti-nutrient content in raw sample of *Prunus amygdalus* (Almonds).

assault on cholesterol is its fatty acid composition. The main factors behind the almond's successful assault on cholesterol are its fatty acid composition (Gustafson, 1999).

Evaluation of Some Nutritional Characteristics of Indian almond Nut (Agunbiade and Olanlokun, 2006)

CHAPTER 2

REVIEW OF LITERATURE

2.1 Almonds

The almond tree is a native of the warmer part of Western Asia and of North Africa, but it has been extremely distributed over the warm temperate region of the old world. The almond belongs to the same group of plants as the rose, plum, cherry and peach, being a member of the tribe *prunae* of the natural order *Rosaceae*. The genus *amygdalus* to which it belongs is very closely allied to *prunus* (plum) in which it has sometimes been merged; the distinction lies in the fruit, the succulent part attached to the stone in the plum (known botanically as the coat in the almond which is hard and juiceless of a dingy green tinged with dull red, that when growing it looks not unlike an unripe apricot (Grieve, 1981).

There are two principal varieties of almonds, the bitter almond (*Prunus dulcis var amara*) and the sweet almond (*Prunus dulcis var dulcis*). The former contains a poisonous glycoside, amygdal which readily breaks down into prussic acid and that prevents its use as human food. The latter is grown for its edible nuts which are used as either raw or in confections and puddings (Phychorraphis, 1977).

Almonds have high protein content, fibre content, vitamin and minerals (Gustafson, 1999 and Duxbury, 1989).

Almonds can help lower cholesterol. Specifically, they reduce low-density lipoprotein (LDL) cholesterol, while preserving the beneficial high-density lipoprotein (HDL) cholesterol. One factor behind the almond's successful

Table 2.1: Weight analysis of Indian Almond fruit component parts

Component part	Total Weight	Percentage of Total weight
Whole fruit	32.95 ± 2.02	-
Edible portion	25.38 ± 1.69	77.02
Endocarp nut	7.11 ± 0.42	21.58
Nut	0.45 ± 0.05	1.40

Values are means ± standard error (SE) of 100 randomly picked Indian almond fruits.

Table 2.2: Proximate composition of Indian almond nut in percentage

Dry matter	97.70 ± 3.81
Crude Protein	11.52 ± 1.10
Ash	6.76 ± 0.72
Crude fibre	5.09 ± 0.84
Fat	21.76 ± 1.20
Carbohydrate	54.87 2.80

Values are means ± standard error (SE) of triplicate determinations

Table 2.3: Values of anti-nutrient and free fatty acids of Indian almond

Parameters	Composition %
Phytin	0.25
Phytin Phosphorous	0.07
Phytin Phosphorous/Total	41.18

Phosphorous * 100	
Cyanide	0.04
Tannin	1.82
Oleic Acid	0.65

Values are means of duplicate determinations

Table 2.4 : Mineral contents of Indian almond nut

Phosphorous %	0.19
Potassium %	0.17
Magnesium %	0.25
Sodium %	245.65
Calcium %	845.45
Manganese %	10.24
Zinc %	92.12
Iron %	70.62
Copper %	9.21

Values are means of duplicate determinations

Tannin, phytin and cyanide significantly influence the function and nutritional properties of foods (Maga, 1982).

Almond kernels are nutritious for humans and the outer hulls have been used as a palatable carbohydrate source for dairy cattle. Profits derived from the sale of hulls to feedlots partially offset the cost of hulling and shelling the almond crop (DePeters *et al.*, 2000).

Nuts and especially almonds are very important food products, from a nutritional point of view mainly due to their high content of numerous beneficial nutritive and bioactive compounds such as vitamin E (26.22 mg/100g), arginine (2.44 g/100g), oleic acid (69.4%) and linoleic acids (17.4%), fiber (12.2 g/100g) and other micronutrients and phytochemical compounds (USDA, 2009).

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Yeasts ordinarily do not compete well in mixed populations, and therefore, cause spoilage only under conditions which are favorable for their growth, but unfavorable for growth of most bacteria (Beech *et al.*, 1969).

The most important factors which determine the ability of yeasts to compete with moulds and bacteria are the number and types of contaminating yeasts, available nutrients, pH, redox potential and water activity (Dennis *et al.*, 1980)

Spoilage of fresh fruits and vegetables by yeasts usually result from their fermentative activity rather than degradation of plant tissue with degradative enzymes. Some yeasts are capable of producing such enzymes and cause spoilage (Ingram, 1958).

Yeasts of the genera *Rhodotorula*, *Cryptococcus*, *Sporobolomyces*, *Saccharomyces*, *Candida* and *Pichia*, amongst others, have been isolated from fresh and rotten fruits (Bhadra and Fleet, 2007)

Different yeast species have been identified as possible sources of phytase (Segueilha and Lambrechts, 1993).

Yeasts possess many properties that make them useful for control purposes. Yeasts generally do not produce allergenic spores or mycotoxins as many mycelial fungi do or antibiotic metabolites likely to be produced by bacterial antagonists (Droby and Chalutz, 1994).

Yeasts can grow rapidly on inexpensive substrates in fermenters and are therefore easy to produce in large quantities (Druvefors, 2004).

Yeast cells contain high amounts of vitamins, minerals, and essential amino acids and there are several reports on the beneficial effects of yeast in foods and feeds (Hussein *et al.*, 1996).

Yeast has been isolated from variety of natural sources like leaves, flowers, fruits etc (Spencer and Spencer, 1997).

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Based on data for hexanal content and given the fact that hexanal derives from oxidation of linoleic acid with oxidation rates of fatty acids being approximately 1:10:100:200 for stearic, oleic, linoleic and linolenic acids respectively one would expect a decrease in linoleic acid and oleic acid concentration which are the predominant fatty acids of almonds (O'Keefe *et al.*, 1993).

A report on an increase in linoleic acid of 4.63 and 3.19% with a parallel decrease in oleic acid of 18.67 and 12.54% for peeled whole almonds stored at 20 and 4 °C respectively for 18 months in the dark under a nitrogen atmosphere (Senesi *et al.*, 1991)

A report on an increase in linoleic acid of 1.4 and 0.7% in case of early harvest shelled whole almonds stored at 20 and 4 °C respectively, with a parallel decrease in oleic acid of 1.4 and 0.6% after 6 months of storage under air (Kazantzis *et al.*, 2003).

2.2 Yeast isolation from fruits

Sporobolomyces, *Saccharomyces*, *Candida*, *Pichia* etc. are isolated from fresh and rotten fruits (Fleet *et al.*, 2003).

Yeasts were isolated from rotten fruits of papaya (*Carica papaya* L.), wine grape (*Vitis* sp.), sapota (*Achras sapota* L.), mangosteen (*Garcinia mangostana* L.), strawberry (*Fragaria* sp. L.), guava (*Psidium guajava* L.), fig (*Ficus carica*), mango (*Mangifera indica* L.) and banana (*Musa paradisiaca* L.), using Rose Bengal agar medium with chloramphenicol (Bhadra *et al.*, 2007).

Table 2.5: Identification of yeasts from rotten fruits.

Source	Strains	Nearest Phylogenic relative	Similarity %
Papaya	YSF104	<i>Pichia galeiformis</i>	100
	YSF9	<i>Kluyveromyces maxianus</i>	99
Sapota	YS22	<i>Issatchenkia orientalis</i>	99
	YSF1	<i>Candida tropicalis</i>	100
	YSF7	<i>Candida viswanathii</i>	99
	YSF14	<i>Pichia guilliermondii</i>	100

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Distinct wild yeast species are supposed to be present and associated with different fruits in natural environments (Spencer *et al.*, 1997).

Table 2.6: Yeast Isolate from fruit source and colony morphological data of different yeast isolated

Fruit	Color	Shape	Surface	Elevation	Consistency
Sapota	Chalky white	Filamentous	Granular	Raised	Dry
Papaya	Dull white	Spheroidal	Smooth	Umbo-nate	Butyrous
Banana	Chalky white	Irregular	Wrinkled	Umbo-nate	Dry brittle

2.3 Phytic acid

Reports have showed that phytin was not hydrolysed by the intestinal enzymes and evidence has been accumulating in recent years that its phosphorus is not available (Plimmer, 1913).

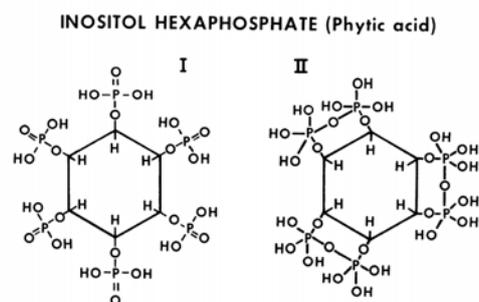


Fig 2.1: Structure of phytic acid (Wheeler, 1971).

Phytin was found to be present in nuts, and in peanuts, cobnuts, almonds and coconuts about half the total Phosphorous was present in this form (Averill and King, 1926).

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Table 2.7 : Phytin content of nuts expressed on a fresh weight basis.

Nuts	Total Pmg per 100g	Phytin P mg per 100g	Phytin P as % total P
Almonds	442	188	42.5
Brazil nut	592	133	22.4
Chestnuts	74	9	12.2
Coconuts	94	41	44.0
Peanuts	365	210	57.5
Walnuts	510	120	23.5

The nutritive contribution of whole grain breads could be increased if the phytates in the products were reduced (Ranhotra *et al.*, 1972)

High phytate content of a food may decrease the absorption of zinc (Reinhold *et al.*, 1973).

Typical hospital diets contain a phytate/zinc molar ratio between 3 and 6 (Harland and Peterson, 1978).

Human studies have indicated that phytate may be hydrolyzed in the gastrointestinal tract (Bitar and Reinhold, 1972).

Some decades ago, it was discovered that phytic acid can form complexes with proteins and several nutritionally important multivalent cations, suggesting that diets rich in phytic acid may impair the absorption of minerals and protein in the digestive tract of humans (Wise 1995 and Plaami 1997).

Moderate amounts of phytic acid are found in artichokes, figs, potatoes, and strawberries and large amounts in cereals, legumes, oil seeds, and nuts (Harland and Harland, 1980).

Phytic acid has a strong ability to chelate multivalent metal ions, specially zinc, calcium and iron. The binding can result in very insoluble salts with poor bioavailability of minerals (Rhou and Erdman, 1995).

Only Inositol triphosphate6 and IP5 have a negative effect on a bioavailability of minerals, the other hydro-lytic products formed have a poor capacity to bind minerals, or the complexes formed are more soluble (Sandberg *et al.*, 1989).

For decades phytate has been regarded as an anti-nutrient, as, during gastro-intestinal passage, it may inhibit the absorption of some essential trace elements and minerals, which under certain dietary circumstances may lead to calcium, iron and zinc deficiencies (McCance *et al.*, 1972).

The main sources of phytate in the daily diet are cereals and legumes, including oil seeds and nuts. They are important for human nutrition and represent 40 and 60% of total caloric intake for humans in developed and in developing countries, respectively (Faostat, 2007).

In nuts, the forth group of phytate-rich food, such as hazelnuts, walnuts, almonds and cashew nuts, the phytic acid content varies by 0.1–9.4% (Harland, 2001).

Table 2.8: Phytate content in nuts

Nuts	Phytic acid/phytate g/100 g
Peanuts	0.17 – 4.47
Almonds	0.35 – 9.42
Walnuts	0.20 – 6.69
Cashew nuts	0.19 – 4.98
Brazil nuts	0.29 – 6.34
Pistachios	0.29 – 2.83
Hazelnuts	0.23 – 0.92

Phytates are the principal storage form of phosphorus and are particularly abundant in cereals and legumes (Reddy *et al.*, 1989).

Phytates chelate divalent cations such as calcium, magnesium, zinc and iron, thereby also reducing their bioavailability (Sandberg, 2002).

Phytates can inhibit the activity of several enzymes and can also reduce the digestibility of legume proteins by forming the complexes with them (Knuckles *et al.*, 1989).

Cereals, legumes, nuts, seeds and tubers are rich sources of phosphorus in the form of phytic acid (myo-inositol hexaphosphate, IP6). This molecule is highly charged with six phosphate groups extending from the central myo-inositol ring and is often reported to be an anti-nutritional factor for humans and animals as it acts as an excellent chelator of cations such as Ca²⁺, Mg²⁺, Fe²⁺ and Zn²⁺. It also complexes the basic amino group of proteins, hindering their absorption and reducing their dietary availability (De Angelis *et al.*, 2003).

Many processes have been reported to remove PA from seeds but the better results are obtained by the use of enzymatic hydrolysis. Phytase (myo-inositol hexaphosphate hydrolase) hydrolyses PA to myo-inositol and inorganic phosphate. It is produced during seed germination or by microorganisms (Urbano G *et al.*, 2000).

The ability of the yeasts strains to degrade phytate was determined on the screening medium. The phytate screening medium (PSM) plates had a cloudy (opaque) appearance due to the presence of calcium phytate while the control plates were transparently clear. Growth after incubation and the production of clear zones around the colonies on the PSM plates were taken as positive for phytate degradation (Morishita *et al.*, 1981).

Phytic acid is a naturally occurring inorganic compound in many cereal grains, oil seeds and legumes. This compound and its derivatives form insoluble complexes with essential minerals such as calcium, magnesium, iron and zinc thereby decreasing the bioavailability of these minerals. The complexes formed may also interfere with enzymatic degradation of proteins. Phytate degrading cultures allowed better access to these nutritionally essential minerals (Torre *et al.*, 1991).

2.4 Fermentation

It was reported that fermentation at 37 °C for 14 h reduced the content of phytic acid in whole-grain pearl millet flour by 43% (Abdalla *et al.*, 1998).

The content of phytic acid decreased by as much as 90% when bran-free pearl millet kernels were fermented at 28 to 30°C for 14 h. After 72 h of fermentation at 30°C, the phytic acid in coarsely ground pearl millet flour was completely hydrolyzed (Khetarpaul and Chauhan, 1990).

Investigated the effects of milling, soaking, malting, heat-treatment, and fermentation on the content of phytic acid in Sudanese sorghum (Mahgoub and Elhag, 1998).

It was found that, after 48 h of fermentation at 30°C, 39% of the phytic acid in a whole grain flour was hydrolyzed (Antony and Chandra, 1998).

Reported that the phytic acid content of finger millet decreased by 60% as a result of germination (24 h) and fermentation (48 h) (Sripriya *et al.*, 1997).

It was reported that the pH of maize dough fell from 6.2 to 3.1 during the 72 h of fermentation (Marfo *et al.*, 1990).

Fermentative reduction in phytate may increase the amount of soluble iron, zinc and calcium several folds (Haard *et al.*, 1989).

Fermentation is one of the oldest and most economical methods of processing and preserving foods. There are many ways to produce foodstuffs by fermenting legumes. Whole or ground seeds, either raw or cooked, can act as substrate for fermentation. The fermented legumes are popular due to improved sensory characteristics, protein quality, starch digestibility and contents of some minerals and vitamins, as well as partial or complete elimination of anti-nutritional factors (Harry *et al.*, 1988).

Fermentation of both wheat and rye brans has been shown to be an efficient pre-treatment method of bran both in order to improve sensory quality of bran-containing bread, and to degrade antinutritive factors, such as phytic acid, in order to improve mineral bioavailability (Hassan *et al.*, 2008).

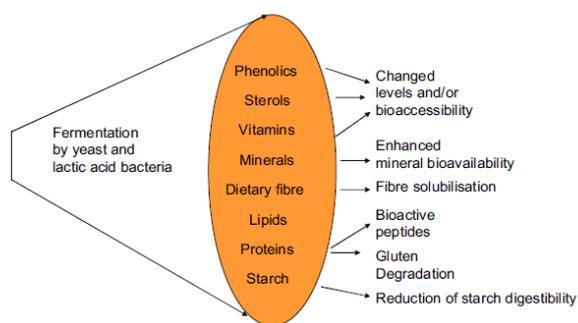


Fig 2.2 Fermentation's influence (Kaisa Poutanen *et al.*, 2009)

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4. The procedure was repeated till the desired dilution was obtained.

3.4.5 Spread Plate Technique

100 μ l aliquots from serially diluted samples were spread aseptically onto the surface of YPD-Ampicillin media (Appendix I) plates using a bent glass rod. Agar plates were incubated in inverted position. The plates were incubated in room temperature ($32 \pm 2^\circ\text{C}$) for 48 hours.

3.5 Staining Of Isolated Microbes

Gram staining (Appendix- II) of isolated microbes was carried out to determine the morphological characteristics and hence confirm the presence of yeast.

3.6 Isolation of Pure, Single Yeast Colony

Quadrant streaking of identified yeast cells was carried out on YPD agar plates to isolate pure yeast cultures.

3.6.1 Quadrant Streaking Technique

1. The inoculating loop was heated to redness, allowed to cool and used to obtain one loopful of broth culture.
2. One edge of the petri plate cover was lifted and the first sector was streaked by making as many streaks as possible without overlapping previous streaks.
3. The loop was heated to redness and allowed to cool, after which the plate was turned. Streaking was carried out through one area of the first sector, and then a few times away from the first sector. The step was repeated to streak the third sector.
4. While streaking the fourth sector, care was taken not to make additional contact with any streaks in the previous sectors.

3.7 Preservation of Isolated Yeast Cultures

Isolated yeast cultures were preserved on YPD slants for frequent usage of culture. Long term storage of yeast cells was carried out by dispersing yeast cells in sterile distilled water and by preparing slants.

3.8 Almond Collection

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CHAPTER 3 MATERIALS AND METHODS

3.1 Glassware

Good quality glassware was used for all tests. All the glassware was of brand Borosil or Riviera. They were washed with good detergent, rinsed in tap water and soaked in chromic acid cleaning solution (Appendix-III).

3.2 Chemicals

Analytical grade chemicals supplied by Loba, Hi- Media, S.D. Fine Chemicals, E.Merck, Qualigens and Sigma Chemicals were used in this study.

3.3 Fruits for Yeast Isolation

Papaya, chikoo and banana were purchased from the local market.

3.4 Isolation of Yeasts

Yeasts were isolated from the ripened pulps of papaya, chikoo and banana. Pulp of fruits like Papaya (*Carica papaya*), Banana (*Musa acuminata*) and Chikoo (*Manilkara zapota*) were taken and mashed in mortar and pestle. The mixture was centrifuged and the supernatant obtained was used for serial dilution.

3.4.3 Serial dilution technique

1. 9ml of sterile distilled water was taken in a series of labelled test tubes for serial dilution. For serial dilution, the first test tube alone contained 10ml of distilled water.
2. 100 μ l of the supernatant was added to the first tube and mixed well. This gave 10^{-1} dilution.
3. 100 μ l of 10^{-1} diluted sample was transferred with a fresh pipette to the second tube and mixed well to give 10^{-2} dilution.

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Commercially available almonds were purchased from the market and labelled as raw sample.

3.9 Processing Of Almonds

200g of each millet sample was washed, dried, powdered and sieved.

3.9.1 Hot Water Extraction Of Almonds And Plating

Almonds were purchased, dried and finely ground and the ground powder was sieved. 2 g of the sieved powder was mixed with 30 ml distilled water. The mixture is kept in boiling water bath for 2-3 hours. The mixture is filtered using cheese cloth. This is autoclaved by after adding 0.75 g of agar. 0.05 g of Ampicillin is added, mixed well and poured on petri plates. A loop full of yeast culture from papaya, chikoo and banana are streaked on individual plates.

3.9.2 Fermentation of almonds:

Almond powders were fermented for 48 hours at room temperature ($32 \pm 2^\circ\text{C}$). 1ml of yeast inoculum was added to the respective media at concentration of 4.5×10^5 cells/ml for papaya and 4.0×10^5 cells/ml for chikoo. pH of the fermented medium was not monitored and the medium was not autoclaved to stimulate household environment.

After fermentation, the slurry was heated at 70°C till the moisture disappeared completely. Thereafter it was finely powdered. The powdered almond samples were stored at 4°C till further analysis.

3.10 Biochemical Analysis of Almonds

3.10.1 Determination of Phytic Acid:

Phytic acid in almond samples was estimated by colorimetric method of Wheeler and Ferrel (1970) that was slightly modified.

The reagents used were as follows

- 3% Trichloroacetic acid (TCA)
- 3% Sodium Sulphate in 3%TCA
- 1.5N NaOH
- 3.2N HNO_3

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- FeCl₃ solution (583mg FeCl₃ dissolved in 100ml of 3% TCA)
- 1.5M Potassium thiocyanate (KSCN)
- Standard Fe(NO₃)₃ solution (100mg Fe(NO₃)₃ dissolved in 100ml distilled water)

Standardization Procedure:

Various volumes of standard Fe(NO₃)₃ solution, ranging from 0.1- 0.5ml was taken in various test tubes and diluted to 4.5ml using distilled water. 2ml of KSCN was added and absorbance was immediately (within 1min) measured at 480nm.

Procedure:

1. Finely ground sample, estimated to contain 5 to 30mg of phytate phosphorous was weighed into a 125ml Erlenmeyer flask.
2. The mixture was extracted with 50ml 3%Trichloroacetic acid with mechanical shaking or occasional swirling by hand for 45min.
3. The suspension was centrifuged and 10ml aliquot of the supernatant was transferred to a 40ml conical centrifuge tube. 4ml of FeCl₃ solution was added to the aliquot by blowing rapidly from the pipette.
4. The contents were heated in boiling water bath for 45min. One or two drops of 3% sodium sulphate in 3% TCA were added when the supernatant was not clear after 30min.
5. The mixture was centrifuged was 10- 15 min and the supernatant was discarded. The precipitate was washed twice by dispersing well in 20- 25ml 3% TCA, heated in boiling water for 5- 10 min and centrifuged. Washing procedure was repeated with water.
6. The precipitate was dispersed in few ml of water and 3ml of 1.5N NaOH was added with mixing. The volume was made to approximately 30ml with water and heated in boiling water for 30min.
7. The mixture was filtered hot (quantitatively) through moderately retentive paper Whatman No. 2. The precipitate was washed with 60- 70ml hot water and the filtrate was discarded.
8. The precipitate from the paper was dissolved with 40ml hot 3.2N HNO₃ into a 100ml volumetric flask. The paper was washed with several portions of water and the

washings were collected in the same flask. The contents of the flask were cooled to room temperature and diluted to volume with water.

9. 1ml of aliquot was transferred to a test tube and diluted to 4.5ml using distilled water. 2ml of 1.5M KSCN was added and colour was read immediately (within 1min) at 480nm.

Calculation:

The µg iron present in the test sample was determined from the standard curve and phytate phosphorous was calculated as per the following equation,

$$\text{Phytate P mg/100g sample} = \frac{\mu\text{g Fe} \times 15}{\text{Weight of sample (g)}}$$

Thus all these studies motivated us to carry out an experiment to reduce the anti-nutrient (Phytic acid) in almonds, using yeast fermentation process hence enabling us to obtain maximum nutritional benefit from almonds.

CHAPTER 4

RESULTS AND DISCUSSION

Results

Yeast spp. were isolated from the pulps of fruits such as papaya, chikoo and banana serial dilution methods, quadrant streaking and spread plate methods. The yeasts isolated from chikoo and papaya grew on almond extracts while the yeast obtained from banana did not utilize almond as a nutrient source. Hence, further work was carried out with the yeasts isolated from chikoo and papaya. The mid-log phase yeast culture was used to calculate the Colony Forming Units (CFU) and it was found to be 4.0×10^5 for chikoo and 4.5×10^5 for papaya. Fermentation of almonds using the yeasts extracted from chikoo and papaya was carried out. 1ml of yeast inoculum was added to the respective media at concentration of 4.0×10^5 (for chikoo) and 4.5×10^5 (for papaya) cells/ml. The phytic acid content in the dried and powdered fermented almond samples were reduced upto 31.9% in chikoo yeast fermented samples and 12.44% in papaya yeast fermented samples.

Discussion

Feil *et al.* (2008) had reported the harmful effects of Phytic acid in human nutrition and the need for reduction of anti-nutrients. Also Harland *et al.* (1980) had explained that the anti-nutrients inhibit the absorption of essential minerals. Ranhotra (1972), Reinhold (1975) and Ter-Sarkissian *et al.* (1974) had stated that the nutritional value of food stuff can be increased if the phytate in the product was reduced. Reddy and Pierson, (1994) had said that the potential health hazards associated with dietary phytic acid aroused interest in the effects of food processing on the content of phytic acid in food.

4.1 Isolation and serial dilution of yeast spp. from fruits

Yeast strains inoculated from serially diluted samples were plated and individual colonies were visible after 48 hours of incubation.

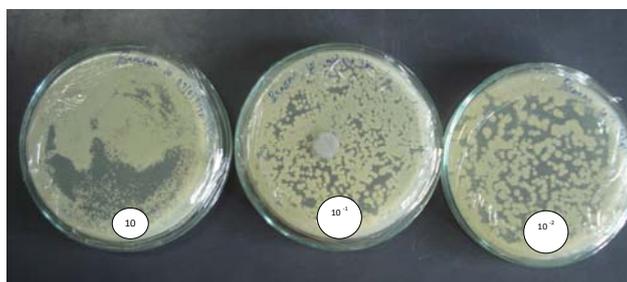


Fig. 4.1 Serial Dilution of sample from Banana

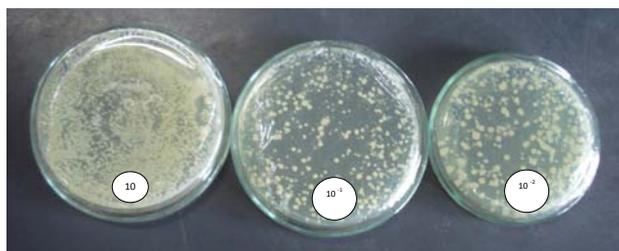


Fig. 4.2 Serial Dilution of sample from Chikoo

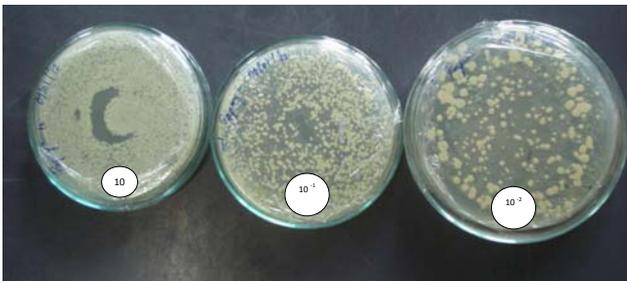


Fig. 4.3 Serial Dilution of sample from Papaya

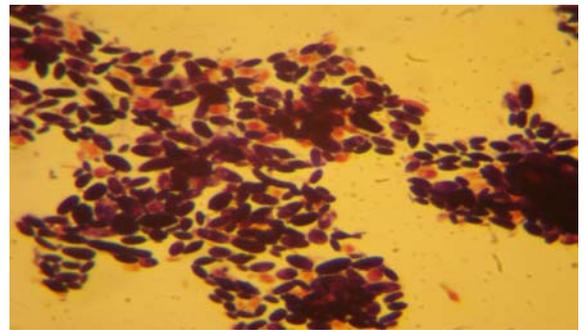


Fig.4.5 Yeast from Chikoo sample

4.2 Isolation of individual colonies

Yeast spp. were isolated and viewed after Gram Staining under the microscope.

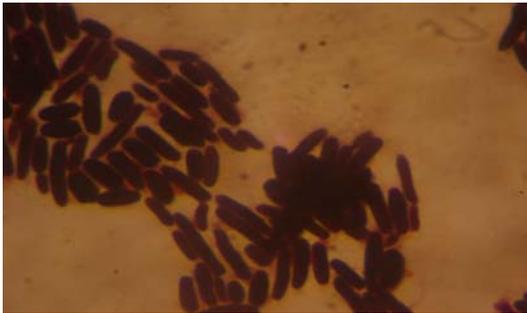


Fig. 4.4 Yeast from Banana sample

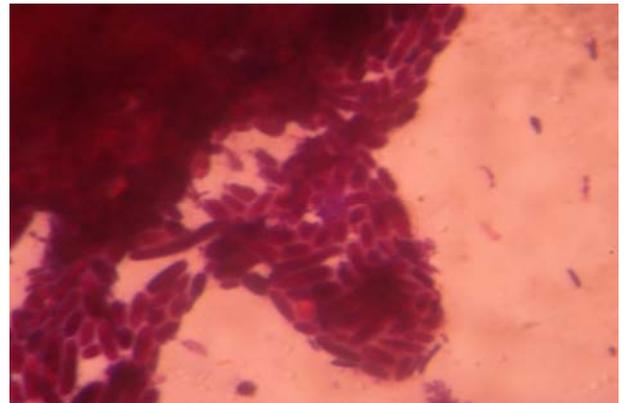


Fig. 4.6 Yeast from Papaya sample

4.3. Growth of Yeast in Almond Medium Prepared By Hot Water Extraction



Fig.4.7 Growth of yeast spp. in almond medium

4.5 Fermented sample

The fermented samples were sealed in air tight bags to prevent yeasts from replicating.



Fig 4.9: Fermented sample

4.5 Colony forming units

Fig 4.8: 100 µl of the diluted yeast culture is plated on agar to determine the CFU (Colony Forming Units) after 48 hours of incubation

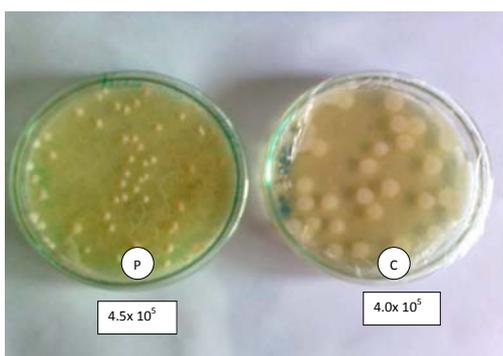


Fig 4.8: Colony Forming Unit (CFU)

4.6 Phytic acid content determination

Table 4.1 Absorbance of almond samples at 480 nm

Raw	Fermented sample using yeast from Chikoo	Fermented sample using yeast from Papaya
0.148	0.302	0.389

4.6.2 Calculation of phytic acid content

From the standard graph (Appendix-IV): $y = 0.0003x - 0.0006$
 $\mu\text{g Fe for samples} \Rightarrow \text{OD at } 480 \text{ nm} = 0.0003x - 0.0006$

1) Raw sample

$$0.148 = 0.0003x - 0.0006 \quad \text{Hence } x = 495.33 \mu\text{g}$$

2) Fermented sample using yeast from CHIKOO

$$0.302 = 0.0003x - 0.0006 \quad \text{Hence } x = 1008.66 \mu\text{g}$$

3) Fermented sample using yeast from PAPAAYA

$$0.389 = 0.0003x - 0.0006 \quad \text{Hence } x = 1298.66 \mu\text{g}$$

Formula:

$$\text{Phytate P mg/100g sample} = \frac{\mu\text{g Fex15}}{\text{Weight of sample (g)}}$$

For RAW sample: 1851.48 mg/100g

For fermented sample from CHIKOO: 1260.82 mg/100g

For fermented sample from PAPAYA: 1623.32 mg/100g

4.7 Phytic acid present after fermentation process

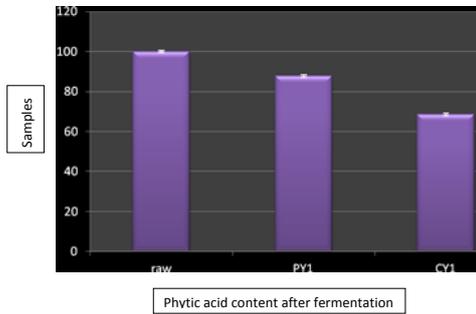


Fig 4.10 Phytic acid present after fermentation process

PY1 => Phytic acid in sample fermented using Yeast from Papaya = 87.56 %

CY1 => Phytic acid in sample fermented using Yeast from Chikoo = 68.10 %

Fermentation process using yeast obtained from chikoo and papaya resulted in the reduction of phytic acid content that was originally present. The amount of phytic acid present in almonds fermented using yeast obtained from chikoo is 68.10% and the almonds fermented using yeast from papaya is 87.56%.

4.8 Reduction in phytic acid content

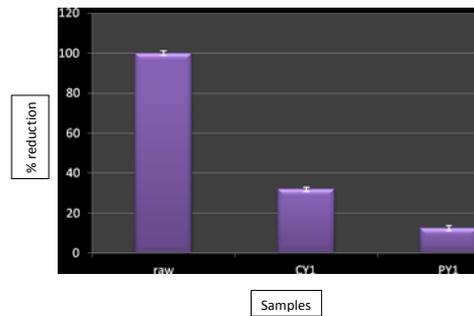


Fig 4.11: Reduction in phytic acid content

CY1=> Reduction of phytic acid in sample fermented with Yeast from Chikoo = 31.90%

PY1=> Reduction of phytic acid in sample fermented with Yeast from Papaya = 12.44%

The reduced phytic acid content after fermentation using yeast obtained from chikoo and papaya is 31.9% and 12.44%.

CHAPTER 5

CONCLUSION

Yeast species were isolated from pulps of fruits like banana, papaya and chikoo by serial dilution process and were identified using Gram Staining method. They were tested for growth in a medium of almond that was prepared by the hot water extraction method. The phytic acid standardization was carried out in order to measure the phytic acid content in samples. The yeast spp. that were isolated from fruits were used as the inoculums for the fermentation process. Fermentation was carried out using YPD broth and the yeast spp. served as the inoculums. The Colony Forming Units (CFU) was also determined. The phytic acid content in raw almonds, fermented samples using yeast strains from Chikoo and Papaya were determined. It was concluded that the Phytic acid content was considerably reduced in the samples that were fermented using the yeast spp. from Chikoo and Papaya. Thus the almonds that were fermented have reduced anti-nutrient content thus increasing their nutritional value.

APPENDICES

Appendix -I: YEAST EXTRACT PEPTONE DEXTROSE (YPD) MEDIUM

Yeast extract	0.5g
Peptone	1g
Dextrose	2g
Ampicillin	0.1g
Agar	2.5g

Appendix-II: GRAM STAINING

Gram staining was carried out by the following procedure:

1. Microbial culture was smeared and heat fixed on a clean microscope slide.
2. Around 10 drops of Crystal Violet stain was added on the microbial culture and left for one minute.
3. Crystal Violet stain was rinsed off with distilled water.
4. Grams iodine was added on the smear and left for one minute, after which it was rinsed off with distilled water.
5. 10 to 15 drops of 95% ethanol was added and left for 10 to 15 seconds after which it was rinsed off with distilled water.
6. Safranin stain was added on the slide and left for 45 seconds after which the slide was rinsed with distilled water.
7. The slide was air-dried and observed under the microscope.

Appendix-III:

Acid Cleaning Solution

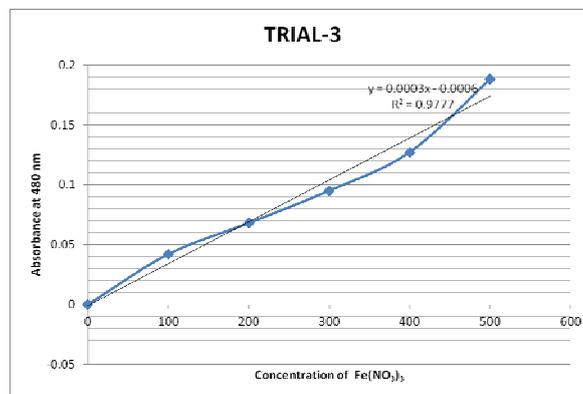
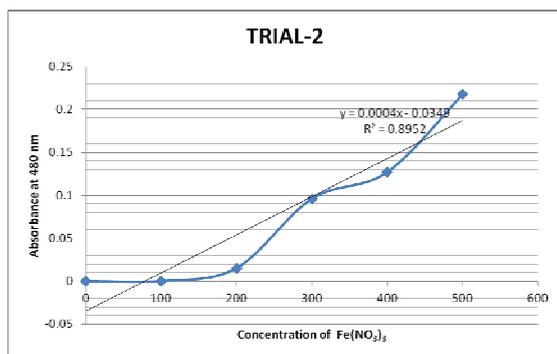
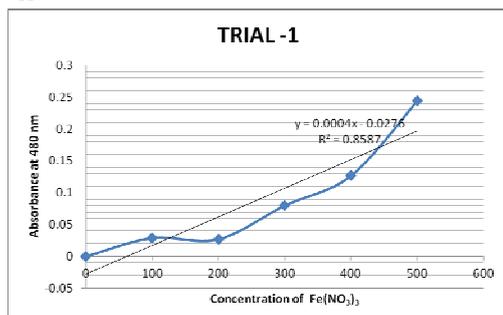
Potassium dichromate	400.0 g
Distilled water	4000.0 ml
Sulfuric acid	400.0 ml

Dissolve potassium dichromate in water. Pour solution into crock, slowly add sulfuric acid. Keeps until solution turns dark brown, depends upon use.

1.5 M Potassium thiocyanate (KSCN)

1.6 Standard Fe(NO₃)₃ solution

Appendix-IV: STANDARDIZATION OF PHYTIC ACID



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