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# CHARACTERIZATION AND APPLICATIONS OF CRUDE α-L-ARABINOFURANOSIDASE FROM SOIL FUNGAL ISOLATES

**A PROJECT REPORT**

***Submitted by***

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**COIMBATORE 641049**

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# KUMARAGURU COLLEGE OF TECHNOLOGY
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## BONAFIDE CERTIFICATE

Certified that this project report "**CHARACTERIZATION AND APPLICATIONS OF CRUDE α-L-ARABINOFURANOSIDASE FROM SOIL FUNGAL ISOLATES**" is the bonafide work of **DEVE M NAIR (0710204010),NITHYA.R(0710204029),PRIYADHARSHINI.Y (0710204034) , SUPRABHA.S (0710204053)** who carried out the project work under my supervision.

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## ABSTRACT

The present work was emphasized on the study of kinetic properties and applications of crude α-L-Arabinofuranosidase from soil fungal isolates. The soil sample collected from the foot hills of Anaikatti, Western Ghats, Coimbatore district was screened for α-L-Arabinofuranosidase producing organisms. The pure colony isolated was identified as *Penicillium* species and the crude enzyme activity was found to be 0.004 U/ml. The studies on kinetic properties of the crude α-L-Arabinofuranosidase has revealed an optimum pH of 5.5, temperature at 55°C and a low $K_m$ of 25μM. The Plackett Burman Design (PBD) screening has showed that the variables like Agitation rate (150 rpm), Orange peel (3.5 %) and $CaCl_2$ (0.1 g/l) were found to have significant effect in the production of α-L-Arabinofuranosidase and the maximum activity was observed as 0.045 U/ml. The Response Surface Methodology (RSM) analysis has confirmed that a combination of high concentration of Orange peel (2.5%), low Agitation Rate (132.95 rpm) and high concentration of $CaCl_2$ (0.75 g/l) yielded a maximum enzyme activity of 0.052 U/ml. Different metal ions like 1mM $Hg^{2+}$ (65%), 1mM $Cu^{2+}$ (44%), 1mM $Mn^{2+}$ (62%) , 1mM $Al^{3+}$(63%) and 1mM $Fe^{3+}$(67%), showed significant α-L-Arabinofuranosidase inhibitory activities. The crude α-L-Arabinofuranosidase enzyme was found to have a noteworthy apple juice clarification and paper pulp effluent degradation properties.

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| | |
|---|---|
| **AF,Araf,α-L-AFases** | α-L-Arabinofuranosidase |
| **AE** | Acetyl Esterases |
| **CCD** | Central Composite Design |
| **EC** | Enzyme Commission |
| **EDTA** | Ethylene Diamine Tetra Acetic acid |
| **FA** | Ferulic Acid |
| **GH** | Glycoside Hydrolases |
| $\mathbf{K_{cat}}$ | Turn over number |
| $\mathbf{K_m}$ | Michaelis – Menten constant |
| $\mathbf{K_{cat}/K_m}$ | Catalytic Efficiency |
| **PBD** | Plackett Burman Design |
| **PNPA, pNPAF** | Para Nitro Phenyl α-L-Arabinofuranoside |
| **RSM** | Response Surface Methodology |
| $\mathbf{V_{max}}$ | Maximum Velocity |

# *Introduction*

# 1. INTRODUCTION

Agro-industrial wastes and byproducts are renewable form of resources generated round the year all over the world. Wheat and rice bran, sugar cane bagasse, corn cobs, citrus and mango peel etc. are one of important wastes of food industries. Industries where such wastes and byproducts are produced are making strict efforts for their proper disposal (Fig 1.1and 1.2). These waste/byproducts, if properly utilized, can widen the economic growth. Advent of biotechnology helped to unlock novel food ingredients through the use of biotechnologically derived industrial enzymes. Their role in bioconversion of waste commodities into value added products has been highlighted in the recent decades. One of the significant applications of agro-industrial wastes like bagasse, fruit peel and wheat bran is biotechnological production of lignocellulolytic enzymes such as cellulases, xylanases, arabinofuranosidases and pectinases. There is an increasing tendency among the people to use chemical free foods. The use of 15 enzymes like xylanases and arabinofuranosidases in the food processing can meet such public demands (Pandey *et.al.*, 2000). Escalating market trend in enzyme fermentation technology has made tremendous progress during the later half of the 20th century. The enzymes thus produced can be utilized in the processing of different foods that is not only advantageous quantitatively but qualitatively as well. Majority of industrial enzymes are produced by large-scale submerged fermentation. Agro-industrial wastes can eventually be used as substrate and act as good sources of carbon and nitrogen. (Rangarajan *et.al.*, 2010).

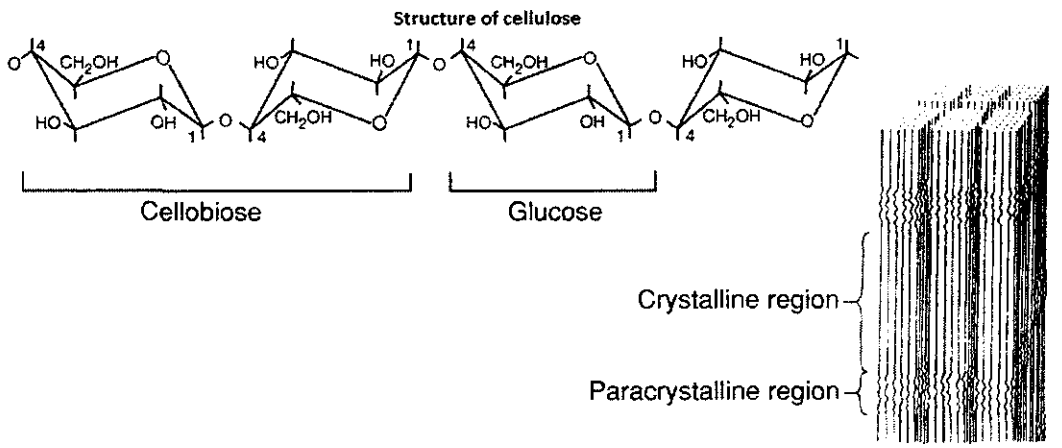
Fig: 1.1 Fruits Waste

Fig: 1.2 Citrus Waste

## 1.1 PLANT CELL WALL POLYSACCHARIDES

Plant cell walls, the major reservoir of fixed carbon in nature, have three major polymeric constituents: cellulose, hemicellulose and pectin. Cellulose is the common component of most plant cell walls (Hoenich, 2006). This macromolecule consists of unbranched, unsubstituted (1, 4)-β-D-glucan chains that are able to form noncovalent microfibrillar complexes through extensive inter- and intramolecular hydrogen bonding and hydrophobic interactions. (Fincher *et.al.*, 2010). They form simple primary and complex tertiary structures. The repeating unit of cellulose is cellobiose. Cellulose can exist in a crystalline or non-crystalline state (Bhat, 2000).The repeating units of cellulose is shown in the Fig 1.3.

Fig: 1.3 Fragment (repeating unit) of a cellulose chain (Bhat et al., 2001)




Cellulose and its derivatives are environmentally friendly, as they are degradable by several bacteria and fungi present in air, water and soil, which are able to synthesize cellulose-specific enzymes (*i.e.* cellulases) (Sannino *et.al.*, 2009). Cellulose has attracted worldwide attention as a renewable resource that can be converted into biobased products and bioenergy. But nowadays, enormous amounts of agricultural, industrial and municipal cellulose wastes have been accumulating or used inefficiently due to the high cost of their utilization processes. Therefore, it has become of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as cheap carbon sources. Cellulose is used as a food source by a wide variety of organisms including fungi, bacteria, plants and protists, as well as a wide range of invertebrate animals, such as insects, crustaceans, annelids, mollusks and

nematodes. These organisms possess cellulases and the complete enzymatic system of them include three different types, that is, exo-β-1,4-glucanases(EC 3.2.1.91),endo-β-1,4-glucanases(EC3.2.1.4), and β-1,4-glucosidase(EC 3.2.1.21). These enzymatic components act sequentially in a synergistic system to facilitate the breakdown of cellulose and the subsequent biological conversion to an utilizable energy source, glucose (Roy *et.al.*, 2009).

Various agricultural residues such as corn fiber, corn stover, wheat straw, rice straw, and sugarcane bagasse, contain about 20-40% hemicellulose, the second most abundant polysaccharide in nature. Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses mannose, glucose, galactose), and sugar acids. Unlike cellulose, hemicelluloses are not chemically homogeneous (Badal, 2003). L-Arabinosyl residues are widely distributed in hemicelluloses such as arabinan, arabinoxylan, gum Arabic, arabinogalactan,etc. (Mondher *et.al.*, 2006). Xylan is the major constituent of hemicellulose. It varies in different plants, from as much as 35 % of the dry weight of Birchwood to as little as 7 % in some gymnosperm. It consists of a backbone of β-1,4-linked xylopyranose and side chains of α,L-arabinofuranoside, an acetyl group, and/or 4-O-methyl glucuronic acid at the C-2 and C-3 positions of the xylose units. Due to the complexity of xylan structure, its complete degradation requires the cooperative action of several enzymes which include xylanase and β-xylosidase, the backbone hydrolyzing enzymes, and debranching enzymes such as α-L-Arabinofuranosidase (AFase), acetyl esterase (AE) and α-glucuronidase (Table 1.1). Numerous enzymes with the ability to hydrolyze hemicelluloses with different linkages have been isolated, most of them from microbial sources (Wagschal *et.al.*, 2007).

**Table: 1.1** Enzymes involved in the hydrolysis of Heteroxylan (Saha, 2000).

| Enzyme | Mode of action |
|---|---|
| Endo-xylanase | Hydrolyzes mainly interior β-1.4-xylose linkages of the xylan backbone |
| Exo-xylanase | Hydrolyzes β-1,4-xylose linkages releasing xylobiose |
| β-Xylosidase | Releases xylose from xylobiose and short chain xylooligosaccharides |
| α-Arabinofuranosidase | Hydrolyzes terminal nonreducing α-arabinofuranose from arabinoxylans |
| α-Glucuronidase | Releases glucuronic acid from glucuronoxylans |
| Acetylxylan esterase | Hydrolyzes acetylester bonds in acetyl xylans |
| Ferulic acid esterase | Hydrolyzes feruloylester bonds in xylans |
| ρ-Coumaric acid esterase | Hydrolyzes ρ-coumaryl ester bonds in xylans |

## 1.2 HEMICELLULOSE AND PECTINS

Hemicelluloses are low molecular weight polysaccharides, which are closely associated with cellulose. They can be removed from the plant cell walls by extraction with aqueous alkali or in some cases with water. Hemicelluloses are classified on the basis of their component sugars. Xylose, mannose, and galactose form the hemicellulose backbone; arabinose, glucuronic acid, and galactose form the side chains (Muhammad, 2010). Furthermore, structural differences exist with one type of polysaccharide isolated from different plant groups or even from different tissues of the same plant

**Table: 1.2** Fruit Cell Wall Composition

| FRUIT | ETHANOL INSOLUBLE RESIDUE % | PECTIN | HEMI CELLULOSE | CELLULOSE | LIGNIN | PROTEINS | TOTAL |
|---|---|---|---|---|---|---|---|
| Apple | 20 | 272 | 169 | 349 | 2 | 76 | 868 |
| Pear | 15 | 281 | 148 | 267 | 69 | 82 | 847 |
| Mango | 25 | 408 | 91 | 236 | 27 | 127 | 889 |
| Pineapple | 13 | 163 | 267 | 210 | 85 | 94 | 819 |
| Strawberry | 12 | 411 | 66 | 232 | 11 | 255 | 975 |
| Raspberry | 20 | 168 | 89 | 177 | 73 | 277 | 784 |
| Cherry | 13 | 396 | 49 | 130 | 169 | 244 | 988 |
| Papaya | 26 | 364 | 165 | 124 | 4 | 127 | 784 |

Pectins are not only important cell wall matrix polysaccharides, but are also present in soft tissues of land plants, such as rinds of citrus fruit (30%), sugar beet pulp (25%), and apples (15%) (Table 1.2). Woody plants contain only minor amount of pectins. The main constituent of pectins is D-galacturonic acid, but various proportions of arabinans and galactans also occur.

## 1.3 ARABINANS AND ARABINOGALACTANS

Arabinans, present in cell-wall in different plant species, have been reported to contain α-L arabinofuranose. They are highly rich in sugar beet, peanut, apple, citrus pectins, carrot, cabbage, rape and mustard seeds. Some arabinans isolated under strongly basic conditions have contained significant proportions of other sugar residues like,D-galactopyranose and galacturonic acid linked to arabinofuranose residues (ElinaLuonteri, 1998). Arabinogalactans have been classified into three types: arabno-1,4-β-D-galactans, arabino-1,3/6-β-D-galactans and a related but distinct group of cell wall glycoproteins containing arabinose and galactose (Table 1.3).In all these the backbone is composed of D-galactopyranose residues substituted mainly at C-6 by single L-arabinose or D-galactose residues or longer branched arabinans or arabinogalactao-oligomers.

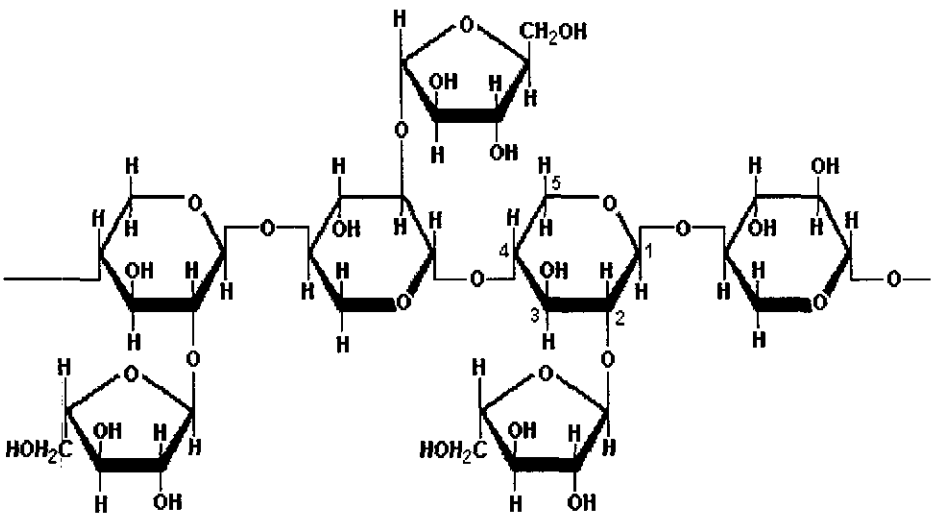
**Table: 1.3** Compositions of Arabinans and Arabinogalactans

| **Polysaccharide** | **Components** | **Linkage** | | **Sources** |
|---|---|---|---|---|
| | | **Main chain** | **Side chain** | |
| Arabinan | α-L-Araf<br>α-L-araf | 1,5 | 1,3<br>1,2 | Rosa glauca<br>Arabinan I DP 100<br>Arabinan II DP 34 |
| Arabino-1,4-β-D-galactan | β-D-Gal*p*<br>α-L-Ara*f* | 1,4 | 1,6<br>1,5<br>(1,3)<br>(1,2) | Potato Gal: Ara 6.4<br>Onion Gal: Ara 9.5<br>Soy Gal: Ara 1.5<br>Citrus Gal: Ara 0.2 |
| Arabino-1,3/6-β-D-galactan | β-D-Gal*p*<br>α-L-Ara*f* | 1,3 | 1,6<br>1,5<br>(1,3)<br>(1,2)<br>1,5<br>(1,3)<br>(1,2) | Coffee bean Gal: Ara 2.7<br>Stractan Gal: Ara 4.9<br>Larch Gal: Ara 2.6-7.8<br>MWs 37000-100000;<br>7500-18000<br>Pine, spruce, doughlas fir<br>Gal: Ara 7-13<br>Exudate gums Gal: Ara<br>0.3-12 |

## 1.4 ARABINOXYLANS

Arabinose containing xylans are found mainly as the secondary wall components in gymnosperms and monocotyledonous plants. Cereal and grass arabinoxylans have a more complex structure than soft wood arabinoglucuronoxylan. Although they contain less uronic acids, they are more highly branched with L-arabinofuranosyl side groups (Fig 1.4). Some ferulic acid (FA) esterifies arabinose at the O-5-position.Other substituents, such as glucuronosyl residues, and short oligosaccharide side chains can also be present but are usually found in low abundance. The fine chemical structures of cell wall arabinoxylans are subjected to modification during normal plant growth and development; many of the changes that occur involve the removal of arabinofuranosyl substituents from the (1-4)-β-xylan backbone (Fincher *et.al.*, 2001).

**Fig: 1.4** Structure of Arabinoxylan.



Cellulose, hemicelluloses like arabinoxylans, lignin and pectins are the key components in the degradation of lignocelluloses. The presence of the side chains restricts the enzymatic hydrolysis of hemicelluloses and pectins. Further, it also represents a formidable technological barrier that retards the development of various industrial processes (Mondher *et.al*, 2006).

Hence, the total biodegradation of xylan is essential for any industrial processes. The complete biodegradation of xylan requires endo-β- 1, 4-xylanase, β-xylosidase, and several accessory enzymes,such as α-L-arabinofuranosidase,α-glucuronidase, Acetylxylan esterase, ferulic acid esterase, and p-coumaric acid esterase, which are necessary for hydrolyzing various substituted xylans (Saha, 2003). Removal of the arabinofuranosyl residues from arabinoxylans is

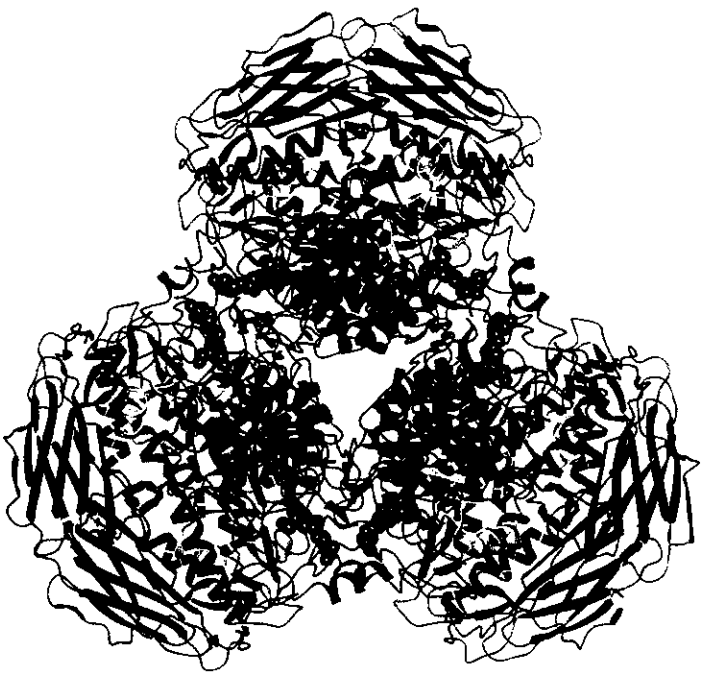
generally attributed to the α-L-arabinofuranoside arabinofuranohydrolases or α-L-Arabinofuranosidase (EC 3.2.1.55) that are most commonly found in rumen or saprophytic micro-organisms; however, these enzymes are also found in higher plants (Fincher *et.al.*, 2001). In this connection, α-L-Arabinofuranosidase becomes very important in the effective use and structural analysis of hemicelluloses (Kusakabe *et.al.*, 1994).

## 1.5 **ARABINOFURANOSIDASE**

α-L-Arabinofuranosidases (Fig 1.5) ,the exo-type enzymes that (AFs; α-L-arabinofuranoside arabinofuranohydrolase; EC 3.2.1.55) catalyze the hydrolysis of nonreducing terminal α-L-arabinofuranosidic linkages in arabinoxylan, L-arabinan, and other L-arabinose containing polysaccharides (Macchabe and Polania, 2007). These enzymes can hydrolyze α-1, 2, α-1, 3 and α-1, 5-arabinofuranosyl linkages of arabinan. The α-L-ArabinoFuranosidases are part of the microbial xylanolytic systems required for the complete breakdown of heteroxylans (Saha *et.al.*, 1998).

Recently, α-L-Arabinofuranosidases have received much attention because of their practical applications in various biotechnological processes, such as, clarification of fruits and vegetable juices, the efficient conversion of hemicellulosic biomass to simple fermentable sugars for subsequent production of fuels and chemicals, delignification of pulp, efficient utilization of plant materials for animal feeds, and hydrolysis of grape monoterpenyl glycoside during wine fermentation (Pinphanichakarn and Lauruengtana, 2006). Reports have shown that, white-rot basidiomycetes are capable of degrading these complex structures (Hemicellulose, pectins and arabinans) efficiently. However, the degree of degradation largely depends on the environmental conditions and the fungal species involved. The study was focused mainly on the characterization and degradation capability of the crude α-L-Arabinofuranosidase from fungal isolates.

**Fig: 1.5** 3D structure of a family 51 Arabinofuranosidase from *Clostridium thermocellum* (Taylor *et.al.*, 2006).



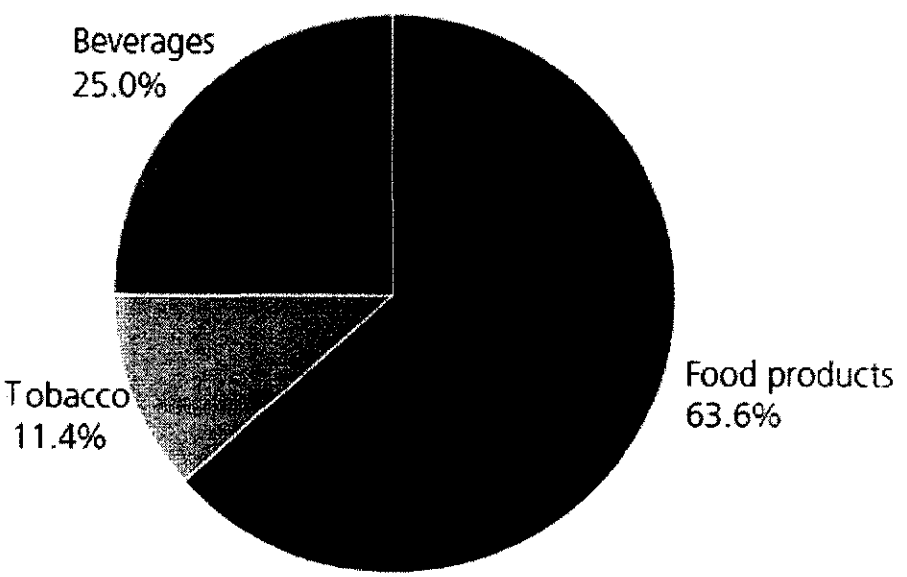
# *Literature Review*

## 2. LITERATURE REVIEW

### 2.1 Food Industries

India is the world's second largest producer of food next to China, and has the potential of being the biggest with the food and agricultural sector. The total food production in India is likely to double in the next ten years and there is an opportunity for large investments in food and food processing technologies, skills and equipment, especially in areas of Canning, Dairy and Food Processing, Specialty Processing, Packaging, Frozen Food/Refrigeration and Thermo processing. Fruits and Vegetables, Fisheries, Milk and Milk Products, Meat and poultry, Packaged/Convenience Foods, Alcoholic Beverages and Soft Drinks and Grains are important sub- sectors of the food processing industry (Deloitte, 2009). Health food and health food supplements is another rapidly rising segment of this industry which is gaining vast popularity amongst the health conscious.The market size of global food, beverage and tobacco products was estimated to be US $ 4,140.3 billion in 2005(Fig 2.1). The size of the global market is around $ 3660 billion in 2005 (not considering tobacco industry which constitutes about 11%) and estimated to grow upto $ 4320 CAGR around 3.35%.

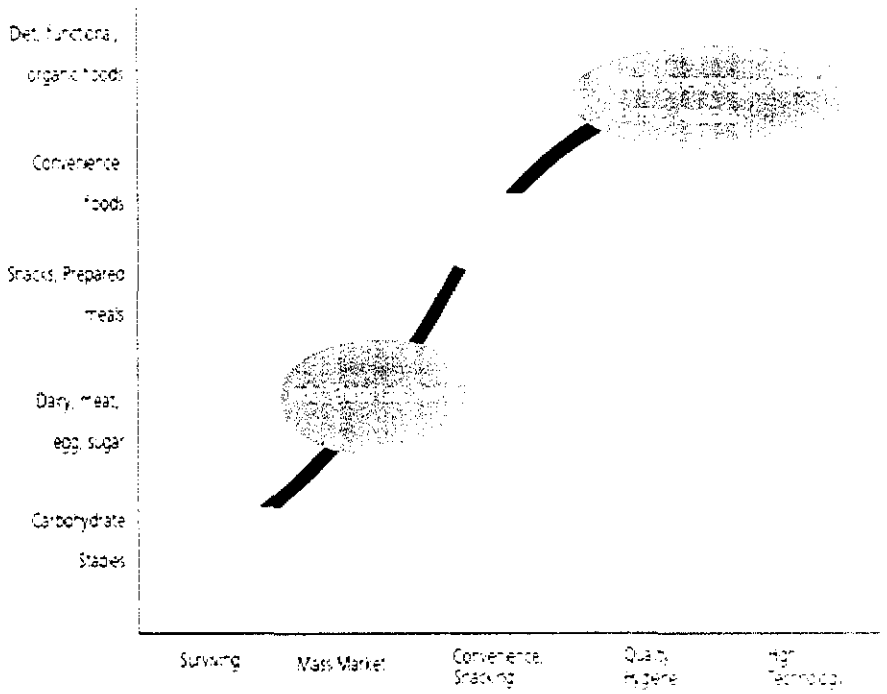
**Fig: 2.1** Global food and Beverage Sector – Category wise contribution



Global market for the processed foods follows the economic power of the countries. Developed economies show more inclination towards processed foods due to higher income levels. Rapid urbanization and rising income levels in the developing economies create the demand for processing foods. Low income levels and poor economic growth of the least developed countries create the demand for basic staples and carbohydrates.

The market can be classified in to four major segments depending on the level of processing and the maturity of the market (Fig 2.2).Countries like USA, Japan and Australia demand highly organic and functional foods whose preparation involves high technology. Quality and hygiene factors are the drivers in the Eastern European countries. Developing countries like India, China and Latin America focus primarily on snacks, prepared meals and processed meat. Carbohydrates still constitute the major food in the least developed markets. Most of the least developed countries are net importers of food.

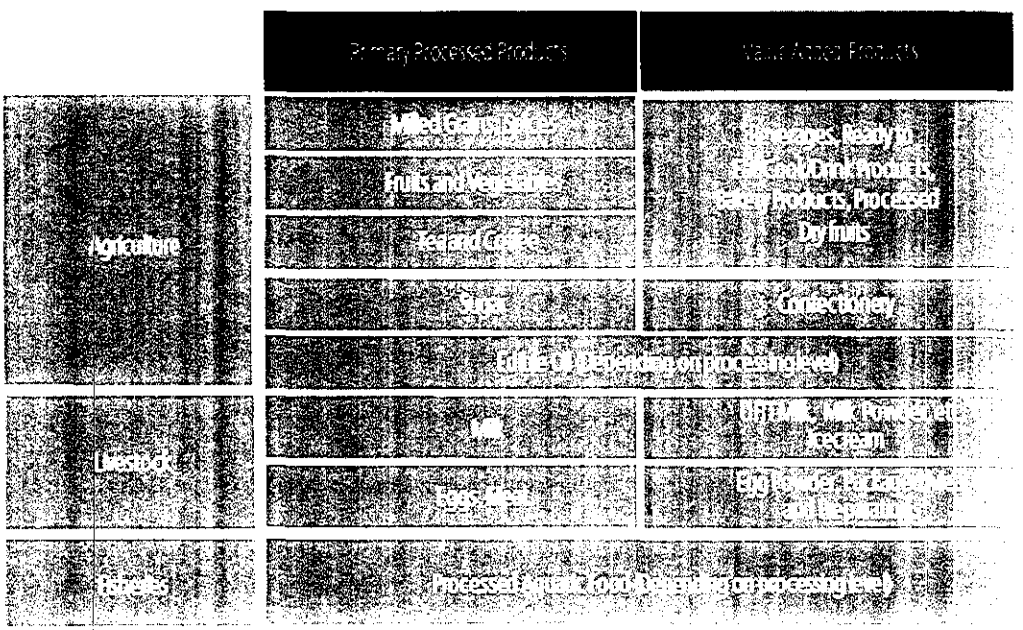
**Fig: 2.2** Evolution of Global Food Demand



### 2.1.1 Primary processed products and Value added products

Includes, cleaning, grading, sorting and packaging. The products manufactured generally act as inputs for value added products (Table 2.1). Manufacturing these products involves use of processing techniques like blending, high temperature heating & boiling, chilling, etc. where the use of technology is significant.

**Table: 2.1** Food products classified into primary and value added products based on the value addition



### 2.2 Developed markets are characterized by high demand for the processed foods

The food processing industry in the USA is considered as a reference for the developed market. There is a very high level of consumption of the processed foods. Nearly 70% of them are highly value added. More than 80% of the food products sold in the USA is packaged and have some brand association. Increasing trend is observed in "Hygienic", "Better for you", "Organic" and "Nutraceutical" products". Although the penetration of the processed foods is very high, the growth rate observed for processed foods is not significant but only about 2.4%.

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**2.3 National scenario of food industries**

In developing countries like India, the share of the processed foods is low compared to that in the developed markets (Fig 2.3). Non-processed foods account for nearly 50% of the share. High value added products account for only 18% of the total share in India. The total size of the Indian food industry is around US$220 billion in 2005. Of that, primary processed food is around $70 billion and the value added segment is about $40 billion. The remaining share is accounted by non-processed food (commodity based). Annual growth rate of the industry is around 9~12%. The wastage levels in India are significant (Table 2.2). Employs around 2 million people (as of 2005).

**Fig: 2.3** Consumption Share – Geographic Distribution.

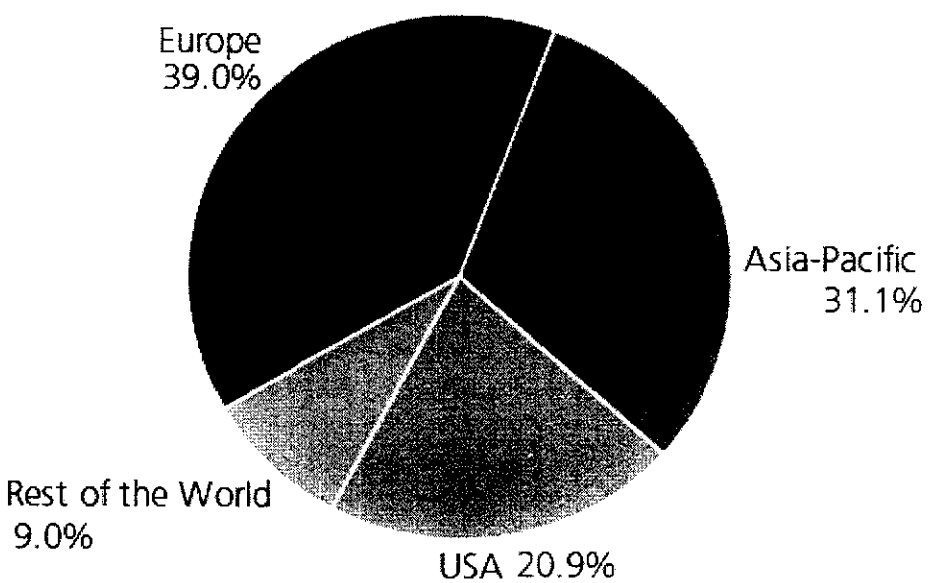


**Table: 2.2** Wastage levels in India

| Commodity | Present level of production | | | Post-harvest losses | | |
|---|---|---|---|---|---|---|
| | Quantity (mn ton) | Average Price (Rs/Ton) | Value (Rs. Crores) | % | Quantity (mn ton) | Value (Rs. Crores) |
| Durables (cereals, pulses and oil seeds) | 230 | 10000 | 230000 | 10 | 23 | 23000 |
| Semi-perishables (potato, onion, sweet potato etc) | 40 | 3000 | 12000 | 15 | 6 | 1800 |
| Perishables (fruits, milk, fish, eggs etc) | 210 | 15000 | 315000 | 20 | 42 | 63000 |
| Total | 480 | 11604 | 557000 | 14.8 | 71 | 87800 |

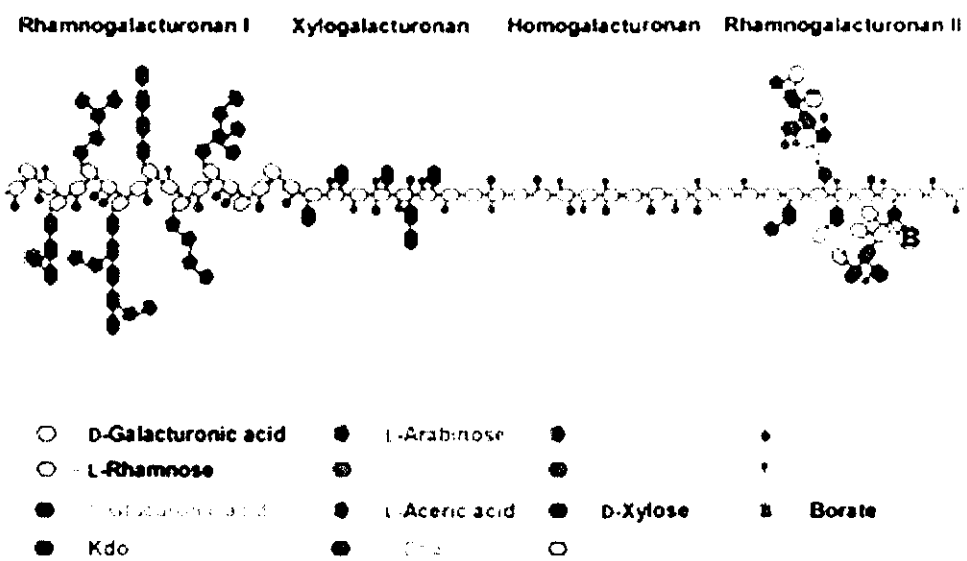
## 2.4 Fruit juice clarification

Fruit juice clarification is one of the major processes carried out in food industry. Different methods for processing of clear juice have been reported with the aim of improving consumer acceptance in the market. Clarification is a beneficial step in the processing of juices, it is often achieved through enzymatic treatment, membrane filtration, or using clarifying aids such as chitosan, gelatin bentonite, polyvinyl pyrrolidone or synergistically combining two clarifying aids compounds. The fruit juice clarification process involves a conventional method. But the conventional fruit juice clarification process, besides having consuming time (12-36 hours) also requires filtration coadjutants and several steps that involve the use of a lot of equipments, as well as the product motion. As a consequence, the costs increase mainly because of the power used, and also due to the possible loss of the product quality. The most promising alternative is membrane concentration. The advantages of reverse osmosis process over traditional evaporation are, in lower thermal damage to product, increase in aroma retention, less energy consumption and lower equipment costs. One main disadvantage of reverse osmosis is its inability to reach the concentration of standard products produced by evaporation, because of high osmotic pressure limitation. The retention of juice constituents, and the permeate flux regarding reverse osmosis performance are two major factors, which are related to the type of membranes and the operating conditions used during the process. The permeate flux was affected mainly by transmembrane pressure, followed by temperature and flow rate. Some substances, as the pectin, contributed to the alteration of the operation conditions and to the viscosity. The concentrated fruit juice by reverse osmosis, is usually preceded by enzymes depectinization and clarification (ultra filtration), being obtained a liquid easier to reverse osmosis process. (Gomes

*et.al.*, 2005). The use of commercial pectic enzymes is common in fruit juice processing. The technological advantages of pectic enzyme use have been shown in many studies; they give a more rapid flow of juice, improve juice yields, facilitate filtration, and gave greater clarity. Since the plant cell wall also contain other components like lignin, hemicelluloses like arabinoxylan, other xylanolytic enzymes along with pectinases can be used to achieve better clarification. (Karangwa *et.al.*, 2010)

Cellulose, hemicelluloses like arabinoxylans, lignin and pectins are the key components in the plant cell wall (Fig 2.4). The presence of the side chains restricts the enzymatic hydrolysis of hemicelluloses and pectins. Further, it also represents a formidable technological barrier that retards the development of various industrial processes like fruit juice clarification. (Numan *et al.*, 2006)

**Fig: 2.4** Schematic Structure of Pectin




Hence, the total biodegradation of xylan is essential for any industrial processes. The complete biodegradation of xylan requires endo-β- 1,4-xylanase, β-xylosidase, and several accessory enzymes,such as α-L-arabinofuranosidase,α-glucuronidase, Acetylxylan esterase, ferulic acid esterase, and p-coumaric acid esterase, which are necessary for hydrolyzing various substituted xylans (Saha, 2003).

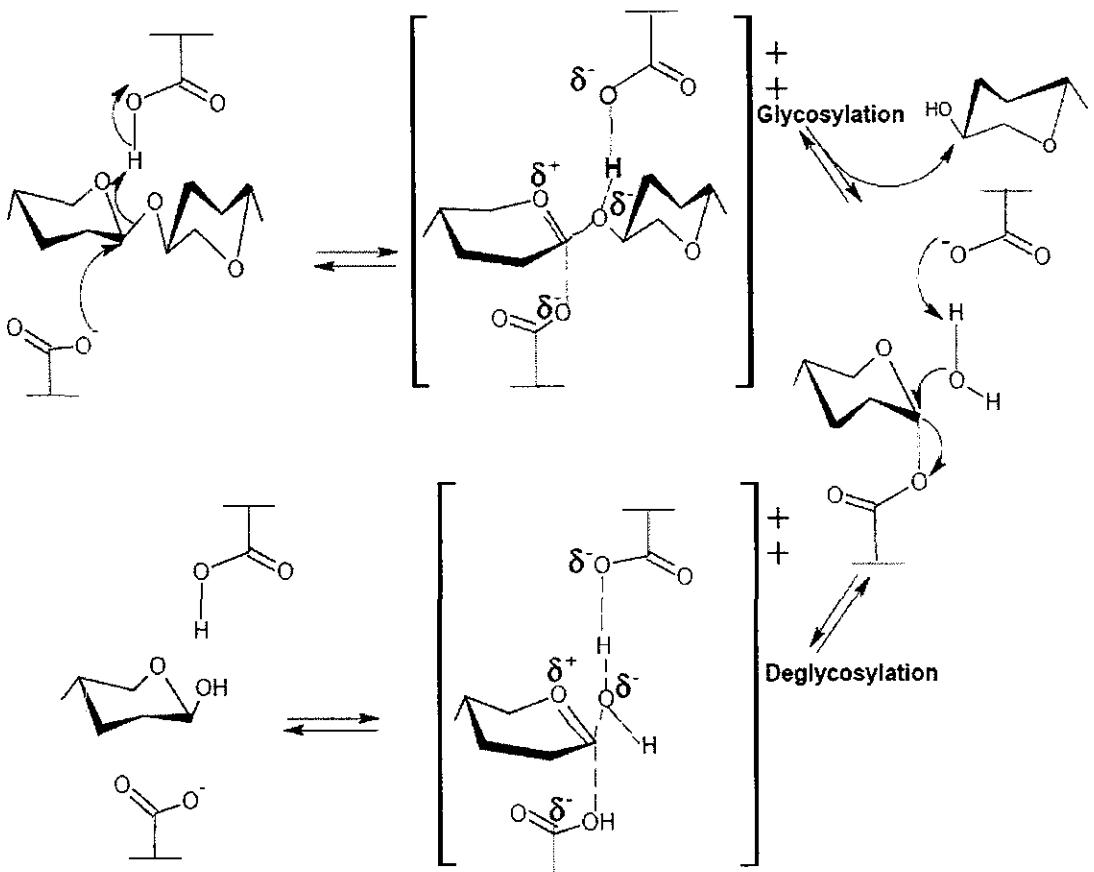
## 2.5 α-L-ARABINOFURANOSIDASE

### 2.5.1 Mechanism of Action

α-L-Arabinofuranosidases,the exo-type enzymes that (AFs; α-L-arabinofuranoside arabinofuranohydrolase; EC 3.2.1.55) catalyze the hydrolysis of nonreducing terminal α-L-arabinofuranosidic linkages in arabinoxylan, L-arabinan, and other L-arabinose containing polysaccharides. (Soham *et.al.*, 1995). These enzymes can hydrolyze α-1,2 ,α-1,3 and α-1,5-arabinofuranosyl linkages of arabinan. The α-L-AFases are part of the microbial xylanolytic systems required for the complete breakdown of heteroxylans (Saha *et.al.*, 1998). Alpha-L-arabinofuranosidases with their synergistic action with other lignocelluloses degrading enzymes are the promising tools in agro-industrial processes. Owing to their industrial importance, a variety of alpha-L-arabino-furanosidases have been purified from various sources such as bacteria, fungi and plants. (Ali, 2009).

Like other glycoside hydrolases(GH), α-L-AFases mediate glycosidic bond cleavage via acid/base-assisted catalysis employing two major mechanisms, giving rise to either an overall retention or an inversion of the anomeric configuration. In both mechanisms the hydrolysis usually requires two carboxylic acids, which are conserved within each glycoside hydrolases family and proceed through an exocarbonium ion-like transition state. Retaining α-L-AFases are members of GH3, GH51and GH54 families that cleave the glycosidic bond using a two-step double-displacement mechanism, as illustrated in (Fig 2.5). In the first step of the reaction (glycosylation), the acid-base residue acts as a general acid, protonating the glycosidic oxygen and stabilizing the leaving group. The nucleophilic residue attacks the anomeric carbon of the scissile bond, forming a covalent glycosyl enzyme intermediate with the opposite anomeric configuration of the substrate. In the second step (deglycosylation), the acid-base residue, acting this time as a general base, activates a water molecule that attacks the anomeric center of the glycosyl enzyme intermediate from the same direction of the original bond, liberating the free sugar with an overall retention of the anomeric configuration.

**Fig: 2.5** GENERAL MECHANISM OF α-L-ARABINOFURANOSIDASE **(Saha, 2003).**




### 2.5.2 Bacterial species

α-L-Arabinofuranosidase has been purified from the extracellular broth of cultures of *Ruminococcus albus*. The molecular weight of the purified enzyme was reported as 75,000Da. The optimum pH was found to be pH 6.9. pI of the enzyme was 3.8. The enzyme yielded a Km of 1.6 mM. The enzyme activity was completely inhibited by 0.05mM of sulfhydryl reagents.0.05 mM $HgCl_2$ or p-mercuribenzoate and 0.01 mM iodoacetamide showed 50% inhibition ( Labavitch *et.al.*, 1984).

α-L-Arabinofuranosidase has been also isolated from *Bacillus subtilis 3-6.* Molecular weight was found to be 61,000Da. The Optimum pH was 7.0 and Optimum temperature was 60°C. The enzyme released arabinose from different substrates (Kusakabe *et.al.*, 1994).

Another α-L-Arabinofuranosidase was isolated from *Bacillus stearothermophilus* T-6. 40% activity is extracellular. Enzyme activity in the cell-free supernatant could reach 25 U/ml. The molecular weight was reported as 256,000Da.Optimum Temperature was found to be 70°C. Optimum pH ranges from 5.5 to 6.0 and pI was 6.5. Thermostability at pH 7.0 was characterized by half-lives of 53, 15, and 1 h at 60, 65, and 70°C, respectively. Kinetic experiments at 60°C with *p*-nitrophenyl a-L-arabinofuranoside as a substrate *V*max - 749 U/mg , *Km* 0.42 mM , and an activation energy -16.6 kcal/mol. The enzyme activity was strongly inhibited by 1 mM $Hg^{2+}$. α-L-arabinofuranosidase released L-arabinose from arabinan and had low activity on oat spelt xylan (Shoham *et.al.*, 1995).

Even α-L-Arabinofuranosidase was isolated from *Thermomonospora fusca* BD25.The main carbon and energy sources used were oat spelt xylan. The substrate used was Para Nitro phenyl α-L-Arabinofuranoside. Specific activity was found to be 0.35 U/mg protein. Optimum temperature of α-L-Arabinofuranosidase lost 10 % of its maximum activity at 55 °C. α-L-Arabinofuranosidase activity remained 83 % of their maximum activity after 9 h of incubation at 50 °C. Maximum relative activity was found to be 0.136 U $mg^{-1}$ protein at pH 9.0.With 54 %and 55 % activities remaining at pH of 4.5 and 11, respectively. Km value was 0.18 mM of p-NPA, Vmax was 0.04 mmol p-nitrophenol $ml^{-1}$ $min^{-1}$. A 15 % reduction in α-L-Arabinofuranosidase activity was detected when L-arabinose (10 mM) was added to the reaction mixture (Tuncer, 2000)

Reports also indicated that α-L-Arabinofuranosidase isolated from *Thermotoga hypogeal* has a Molecular weight of 92KDa for Homodimer and for Tetramer it was 50KDa.The Substrate used was 4-nitrophenyl-α-Larabinofuranoside.The enzyme was stable at 40°C with a 10 minutes incubation time. The Optimum Temperature was found as 40°C and the Optimum pH was 6.0. The Specific activityof the enzymes varied in accordance with the substrate used (Salma, 2008).

A gene encoding an α-L-arabinofuranosidase, designated SaAraf43A, was cloned from Streptomyces avermitilis. The recombinant enzyme was isolated.Optimum pH was found to be 6.0 and Optimum Temperature was reported as 45°C. pH range of 5.0–6.5 and at temperature of 30°C. The enzyme hydrolyzed p-nitrophenol (PNP)-α-L-arabinofuranoside.The enzyme only cleaves α- 1,5-linked arabinofuranosyl linkages. The enzyme releases arabinose in exo-acting manner. These results indicate that the enzyme is definitely an exo- 1,5-α-L- arabinofuranosidase (Ichinose, 2008)

### 2.5.3 Fungal species

An extracellular ('E') and intracellular ('I') α-L-Arabinofuranosidase isolated from *Sclerotinia fructigena* showed the following substrate utility and kinetic properties. Substrate used was *25* mg of p-nitrophenyl-α-L-arabinofuranoside. ' E' enzyme was stable over the pH range 4 to 7, but 'I' was stable only at pH 6 to 7. Maximum activity for 'E' was recorded at pH 4-5, and that for 'I' at pH 5.5 to *6.0*. Results of Lineweaver-Burk plot indicates the optimum $K_m$ values for 'I' 0.28 mM at pH 4.5 and 0.23 mM at pH 5.5, and for 'E' its 0.66 mM at pH 4.5 and 1.1 mM at pH 5.5( Laborda *et.al.*, 1973).

Another α-L-Arabinofuranosidases (Arafs) I and II were purified from the culture filtrate of the fungi *Aspergillus awamori* IFO 4033. Molecular weights Araf I and II were reported as 81,000 Da and 62,000 Da respectively. Both enzymes exhibit higher activity at pH 4.0. pIs of the enzyme were found to be 3.3 and 3.6. Optimum temperature was found to be 60°C and The enzyme was almost stable at pH values from 3 to 7 and at temperature up to 60°C.The enzymes released significant amount of arabinose from different substrates (Kusakabe *et.al.*, 1998).

α-L-Arabinofuranosidase was also isolated from A color-variant strain of *Aureobasidium pullulans* (NRRL Y-12974). Molecular weight of the enzyme was reported to be 210,000Da and the enzyme had a half-life of 8 h at 75°C. This shows that the fungal enzymes are highly themostable.Optimum Temperature was reported as 75°C.Optimum pH ranges from 4.0 to 4.5. Specific activity of the enzyme was 21.48 mmol/min/mg of protein. The enzyme doesn't require any metal ion for its activity, and hence their activity was not affected by p-chloromercuribenzoate (0.2 mM), EDTA (10 mM), or dithiothreitol (10 mM) (Saha, 1998).

Another α-L-Arabinofuranosidase was isolated from *Thermomyces lanuginosus*. Carbon source used was 2% wheat bran. Optimum Temperature was found to be 70°C. Optimum pH ranged from 4.0 to 4.5.The enzyme was stable at 30-90 °C and pH 3.5-5.5 or at 4 °C for a month. It had a half-life of 12 and 2 h at 70°C and 90 °C respectively (El-Gindy *et.al.*, 2003).

α-L-Arabinofuranosidase isolated from *Aspergillus niger* and *A. Oryzae* indicated the Optimum pH as 5.5 for *Aspergillus niger* and 6.0 for *A. Oryzae*. Optimum temperature was found to be 30°C for *Aspergillus niger* and 35°C for *A. Oryzae*.The enzyme was stable at temperatures around 30-90°C and pH 3.5-5.5 (Ali, 2009).

α-L-Arabinofuranosidase isolated from *Talaromyces thermophilus* clearly indicated the suitable Carbon and the Nitrogen source for enzyme production were wheat bran and yeast extract. The Optimum pH was found to be 7 and the Optimum Temperature was found to be

55°C. The PB design was used to screen the significant variables and they reported that pH, wheat bran and MgSO4 has maximum effect on enzyme production among pH, inoculum size, wheat bran , yeast extract, agitation rate, aeration, $CaCl_2$ and $MgSO_4$ in T. thermophilus. optimum levels of these significant factors and the effect of their interactions on α-L-arabinofuranosidase production were also studied and reported ( Guerfali *et.al.*, 2010).

A gene of α-L-arabinofuranosidase (Abf) from *Trichoderma koningii* G-39 was successfully expressed in *Pichia pastoris*. Substrate used was p-nitrophenyl-α-L-arabionfuranoside (pNPAF). The enzyme was stable for at least 2 h at pH values between 2 and 8.3 at room temperature. Enzyme activity decreased dramatically when the pH exceeded 9.5 or dropped below 1.5. The enzyme lost 35% of activity after incubation at 55°C for 2 h. (Li *et.al.*, 2007)

**2.5.4 Plant species**

α-L-Arabinofuranosidase was isolated from barley (Hordeum vulgare L) .The molecular mass was reported as 65 kDa. Substrate used was cereal cell wall arabinoxylans. The Optimum pH was found to be 4.3 and pI was found to be 4.8. kcat was found as 6.9.kcat/*K*m was 0.76 (Fincher *et.al.*, 2001).

Another α-L-Arabinofuranosidase was isolated from Strawberry fruit (fragaria x ananassa). The Substrate used was 4-nitrophenyl-a-Larabinofuranoside. 0.05% (v/v) Triton X-100 showed some inhibition of the enzyme activity and 1 mmol of $ZnCl_2$- showed no inhibition (Martinez *et.al.*, 2009).

To investigate the role of α -L-arabinofuranosidase the enzyme in the xylem vessel formation in *Arabidopsis thaliana*, the recombinant protein was expressed in *Pichia pastoris* and the properties were characterized. Optimum pH was reported to be 4.5and Optimum Temperature was found to 50°C. The enzyme was stable over the pH range of 4.0–7.0 under 30°C.Substrate: The enzyme released L-arabinose from *p*-nitrophenyl-a -L-arabinofuranoside, synthetic arabinofuranobiosides, arabinoxylo-oligosaccharides and arabinose containing polysaccharides. The enzyme hydrolyzed *p*-nitrophenyl-a -L-arabinofuranoside but did not hydrolyze any other *p*-nitrophenyl-glycosides.Specific activity for *p*-nitrophenyl-a -L-arabinofuranoside was 1.2 units $mg^{-1}$. Among the synthetic regioisomers of arabinofuranobiosides, the enzyme hydrolyzed all linkages that can occur between two α –L-arabinofuranosyl residues in the following order: α-1, 5-linkage>α -1, 2-linkage>α -1, 3-linkage.

The enzyme hydrolyzed arabinan, gum arabic, corn hull arabinoxylan, and wheat arabinoxylan (Kaneko *et.al.*, 2010).

## 2.6 APPLICATIONS

a) **Production of arabinose by α-L-Arabinofuranosidase**

α-L-Arabinofuranosidases are capable of releasing arabinose from different agricultural raw materials which can act as an antiglycemic agent as for its importance in food industry (Viikari *et.al.*, 1994).

b) **Production of antimetastatic compounds by α-L-Arabinofuranosidase**

Ginsenosides Rb2 and Rc are the main components of ginseng (the root of *Panax ginseng* C. A. Meyer, Araliaceae).Ginsenosides Rb2 and Rc are L-arabinofuranoside and L-arabinopyranoside-bound glycosides, respectively. In ginsenoside Rd, these ginsenosides are transformed to compound K, via ginsenoside Rd, by intestinal bacteria in human intestine by the action of α-L-AFase. The latter bacteria utilize α-L-AFase to transform the protopanaxadiol ginsenosides to compound K that exhibits antimetastatic and/or anticarcinogenic effects (shin *et.al.*, 2000).

c) **Role α-L-Arabinofuranosidases in wine industry**

Treating grapes with α-L- Arabinofuranosidases followed by the addition of other glycosidases can be used for the enhancement of wine flavour by the release of free terpenols (Maeto, 2000).

d) **Production of acetic acid by α-L-Arabinofuranosidases**

Pentosans added to the dough may be moderately hydrolysed by α-L-Arabinofuranosidases to give acetic acid which may increase the bread quality (Gobbetti *et.al.*, 2000).

e) **Role of α-L-Arabinofuranosidases in pulp and paper industry**

Application of α-L- Arabinofuranosidase would enhance the delignification of pulp in pulp industry as the enzyme acts to release arabinose side chain that retard the action of other bleaching enzymes (Nakas and Makkonen, 2005).

f) **Role α-L-Arabinofuranosidases in animal feedstock**

Although hemicelluloses (mainly xylans) represent about 30 to 40% of the total forage carbohydrate, their contribution to dietary energy available to the animal is often decreased because of low overall (40to 60%) digestion. The increase in digestibility of feedstuffs is well

correlated with the decrease in the degree of substitution of the hemicelluloses polymers with arabinosyl residues. Therefore, any mechanism able to remove the arabinosyl side chains from hemicellulose should increase its digestibility. The addition of α-L-Arabinofuranosidases remove arabinose side-groups and could further promote the hydrolysis of solubilized cell wall polysaccharides (Shin *et.al.*, 2003).

g) **Role of α-L-Arabinofuranosidases in fruits juice industry**

α-L-AFases are receiving attention for their applications in fruit juice clarification. These enzymes specifically remove the 1,3–side chains present on the main 1,5-linked arabinan chains. This results in a precipitate (haze) consisting of 1,5 arabinans. The α-1,5 arabinanase acts on 1,5 arabinans that help to increase the solubility of the precipitate. The precipitates can most probably be avoided by adding sufficient amounts of α-L-Arabinofuranosidase and endo-arabinanase (Saha, 2000).

h) **Production of fermentable sugars for bioethanol industry by α-L-Arabinofuranosidase**

Enzymes catalyzed conversion of sugarcane, sugar beet, corn or wheat to ethanol by distillers yeast *Saccharomyces cerevisiae* is the currently used process for the industrial production of bioethanol. These substrates contain nonfermentable hemicelluloses. These hemicelluloses remained unutilized and accumulate as by-product residues (~70 wt % of the total residue) during the process of ethanol production. The synergistic action of α-L-AFases with lignocelluloses degrading enzymes makes them potentially most suitable agents for saccharifying various pretreated agricultural and forestry residues to monomeric sugars for the production of fuel and chemicals (Mondher *et.al.*, 2006).

Eventhough α-L-Arabinofuranosidase have been isolated and characterized from many different species,there are lot of scientific documentations to be reported yet. In this concern, our present work has been focussed on characterization and applications of crude α-L-Arabinofuranosidase from the soil fungal isolates.

# *Objectives*

## 3. OBJECTIVES

- To screen for the presence of α-L-Arabinofuranosidase producing fungal species from soil sample.

- To characterize the kinetic properties (pH, Temperature and $K_m$) of crude α-L-Arabinofuranosidase.

- To optimize the media composition using Plackett-Burman Design and Response Surface Methodology.

- To study the effect of inhibitors on the crude α-L-Arabinofuranosidase activity.

- To apply the crude α-L-Arabinofuranosidase enzyme in fruit juice clarification, paper & pulp degradation.

# *Materials and Methods*

# 4. MATERIALS AND METHODS

## 4.1 MATERIALS

p-Nitrophenyl-α-L-Arabinofuranoside (PNPA) was obtained from Sigma Chemical Co., (USA). All other chemicals used in the experiments were of analytical grade.

## 4.2 METHODS

### 4.2.1 SAMPLE COLLECTION AND SCREENING FOR FUNGAL ISOLATES

The soil sample was collected from the foothills of Anaikatti, Western Ghats. About 1g of the soil sample was weighed and dissolved in 100 ml of distilled water ($10^{-1}$). An aliquot of 1ml was transferred to 9 ml of distilled water to make dilution of $10^{-2}$.This was followed till $10^{-6}$ dilution. Then from each dilutions about 0.1ml of the sample was transferred to the Potato dextrose agar plates using spread plate technique. Potato dextrose agar medium was prepared by weighing 3.9g dissolved in 100 ml of distilled water and sterilized in autoclave for 15 minutes at 120°C. The media was then plated evenly in 25 ml petri dish and allowed for solidification. After 48 hrs of incubation the growth of fungal colonies in all the dilutions were observed. Colonies from$10^{-4}$ and $10^{-5}$ dilution were chosen for pure culture isolation. From that only two colonies alone were quadrant streaked and the single colony was isolated, which is then further analyzed for genus identification. This was done using the common fungal stain Lactophenol cotton blue. (1 ml of stain mixed with one loopful of colonies, dried and viewed under Reflected light Fluorescence microscope).

### 4.2.2 SCREENING FOR ENZYME

From the isolated fungal species about one loopfull of colonies ($10^3$cells) were transferred to 100ml Potato Dextrose broth (2.4g in 100ml distilled water and sterilized) and kept under shaking condition (37°C) for one week. The culture filtrate was then centrifuged (cooling centrifuge) at 10,000 rpm for 10 mins and the collected supernatant was used for further enzymatic studies.

### 4.2.3 ENZYME ASSAY

#### 4.2.3.1 Principle

The principle behind the detection of arabinofuranosidase activity is the liberation of yellow coloured product p-nitrophenol. Arabinofuranosidase acts on the substrate p-nitrophenyl-arabinofuranoside resulting in a yellow colored solution which is measured spectrophotometrically at 410nm.The intensity of color depends on the amount of p-nitrophenol released which is directly proportional to the amount of enzyme activity in the sample.

#### 4.2.3.2 Preparation of stock solutions

(i) Citrate buffer

Stock solution of A: 0.1M solution of citric acid (21.01g in 1000ml), and stock solution of B: 0.1M solution of sodium citrate (29.41g in 1000ml) was prepared. Citrate buffer was prepared by mixing appropriate proportion of A and B, diluted to 100ml and adjusted to required pH values.

(ii) substrate stock solution(1mM)

About 0.27mg of PNPA was dissolved in lml of 50mM citrate buffer (pH- 5.0)

#### 4.2.3.3 Assay Procedure

A Slightly modified spectrophotometric method proposed by Poutanen and plus, 1984 and Mohander *et.al.*, 2005 was adopted to determine the activity of crude α-L-Arabinofuranosidase. About 0.2ml of 1mM p-nitrophenyl α-L-arabinofurnoside (PNPA) and 0.1ml of 50mM citrate buffer is added and incubated at 50°C for about 30 mins. The reaction was stopped by adding 0.5ml of sodium bicarbonate. The liberated p-nitrophenol (yellow colour) was measured spectrophotometrically at 410nm. One unit of the enzyme activity was defined as the amount of enzyme that liberated 1µmol of p-nitrophenol per minute under the assay conditions.

$$\text{Enzyme activity (U/ml)} = \frac{\text{(Concentration x Dilution factor x Reaction Volume)}}{\text{(1000 x Incubation Time)}}$$

Dilution Factor= Total Volume/ Enzyme Volume

## 4.3 CHARACTERIZATION

### 4.3.1 Determination of pH optima

The optimal pH was determined in the range from pH 3.0 to 6.0 (50 mM Citrate buffer). The activity was assayed by the same procedure as that of (4.2.3.3).

### 4.3.2 Determination of temperature optima

The optimum temperature for α-arabinofuranosidase activity was determined by incubating the reaction mixture at various temperatures (30-90°C) at pH 5.5 using 50 mM Citrate buffer. The activity was assayed by the same procedure as that of (4.2.3.3).

### 4.3.3 Determination of Km

In the kinetic studies of α-arabinofuranosidase, the substrate PNPA concentration was varied from 20μM to 100μM and the activity of the enzyme was recorded as in (4.2.3.3).

### 4.3.4 Inhibitor studies

The effects of inhibitors on α-arabinofuranosidase activity was also studied using inhibitors like $ZnCl_2$, $Al_2(SO_4)_3$, $FeCl_2$, $NiCl_2$, $CuSO_4$, MnSO4, $CaCl_2$, EDTA, $HgSO_4$. The enzyme activity was assayed as that of (4.2.3.3).

## 4.4 MEDIA OPTIMIZATION

### 4.4.1 PLACKETT BURMAN DESIGN

The variables selected for the present study includes,

(a) pH (4 and 7)

(b) Orange peel (1.5% and 3.5%)

(c) Yeast extract (0.1(g/l) and 1.0(g/l))

(d) Agitation rate (150 and 200 rpm)

(e) Calcium chloride (0.5(g/l) and 1.0(g/l))

(f) Magnesium sulphate (0.5(g/l) and 1.0(g/l))

(g) Innoculum size ($10^5$ and $10^8$ spores/loop)

The experimental runs obtained is show in Table (4.1)

**Table 4.1 Experimental runs (PBD)**

| pH | Incoculam size | Orange peel | Yeast extract | Agitation rate | calcium chloride | magnesium sulphate |
|---|---|---|---|---|---|---|
| 4 | 8 | 3.5 | 1.0 | 150 | 1.0 | 1.0 |
| 4 | 5 | 1.5 | 1.0 | 200 | 1.0 | 0.5 |
| 7 | 8 | 3.5 | 0.1 | 200 | 1.0 | 0.5 |
| 4 | 8 | 1.5 | 0.1 | 150 | 1.0 | 1.0 |
| 4 | 5 | 3.5 | 1.0 | 200 | 0.5 | 1.0 |
| 7 | 5 | 3.5 | 1.0 | 150 | 1.0 | 0.5 |
| 4 | 8 | 3.5 | 0.1 | 200 | 0.5 | 0.5 |
| 7 | 8 | 1.5 | 1.0 | 200 | 0.5 | 1.0 |
| 7 | 8 | 1.5 | 1.0 | 150 | 0.5 | 0.5 |
| 7 | 5 | 3.5 | 0.1 | 150 | 0.5 | 1.0 |
| 4 | 5 | 1.5 | 0.1 | 150 | 0.5 | 0.5 |
| 7 | 5 | 1.5 | 0.1 | 200 | 1.0 | 1.0 |

## 4.4.2 RESPONSE SURFACE METHODOLOGY

RSM was carried out and the combinations of variables chosen are shown in **Table (4.2)**

**Table 4.2** Experimental runs (RSM)

| C5 | C6 | C7 |
|---|---|---|
| Agitation rate | Orange peel | CaCl2 |
| 132.955 | 2.50000 | 0.75000 |
| 175.000 | 2.50000 | 0.75000 |
| 150.000 | 1.50000 | 0.50000 |
| 200.000 | 1.50000 | 1.00000 |
| 175.000 | 2.50000 | 0.75000 |
| 175.000 | 2.50000 | 1.17045 |
| 175.000 | 2.50000 | 0.75000 |
| 200.000 | 3.50000 | 1.00000 |
| 150.000 | 3.50000 | 0.50000 |
| 150.000 | 3.50000 | 1.00000 |
| 175.000 | 2.50000 | 0.32955 |
| 217.045 | 2.50000 | 0.75000 |
| 175.000 | 2.50000 | 0.75000 |
| 175.000 | 4.18179 | 0.75000 |
| 175.000 | 2.50000 | 0.75000 |
| 150.000 | 1.50000 | 1.00000 |
| 200.000 | 3.50000 | 0.50000 |
| 175.000 | 0.81821 | 0.75000 |
| 200.000 | 1.50000 | 0.50000 |
| 175.000 | 2.50000 | 0.75000 |

## 4.5 APPLICATIONS

### 4.5.1 Fruit Juice Clarification

In order to check the efficiency of crude α-L-Arabinofuranosidase in clarification process a think extract was prepared (150 g of apple, cleaned thoroughly cut into small pieces and mixed with 25ml of water and macerated). To this about 1.5 ml of the crude Arabinofuranosidase extract was added and incubated for four days.

Similarly the grape peel was cut into small pieces (2x2cm) and incubated with the 1ml enzyme extract for four days.

### 4.5.2 Paper and Pulp Degradation

Arabinofuranosidase activity in paper and pulp degradation was also studied. About 20 ml of highly viscous effluent was diluted with 20ml of distilled water and added with 5ml of the enzyme extract. The mixture was left undisturbed for 6 days and the results were interpreted.

## 4.6 STATISTICAL ANALYSIS

The statistical analysis for PBD and RSM was carried out using MINITAB 16 Software.

# *Results and Discussion*

# 5. RESULTS AND DISCUSSION

## 5.1 SCREENING

Soil collected from the foot hills of Western Ghats (Annaikkatti) was screened for fungal species. From the different colonies, two colonies were separated and their pure cultures were isolated. By staining, one of the colonies was found to posses certain morphological characteristics similar to *Penicillium* species as shown in the Fig 5.1.The conidiophores of phialides are square shaped. Another colony showed morphological characteristics similar to *Aspergillus* species. The colonies were stained to green colour and a branched hypha was found. The broth culture filtrate analysis has revealed about 0.004 U/ ml and 0.0012 U/ ml of α-L-Arabinofuranosidase activity from *Pencillium* and *Aspergillus* species, respectively. Since the Aspergillus species showed a very low activity of α-L-Arabinofuranosidase , it was neglected and our study was focussed only on *Penicillium* species.

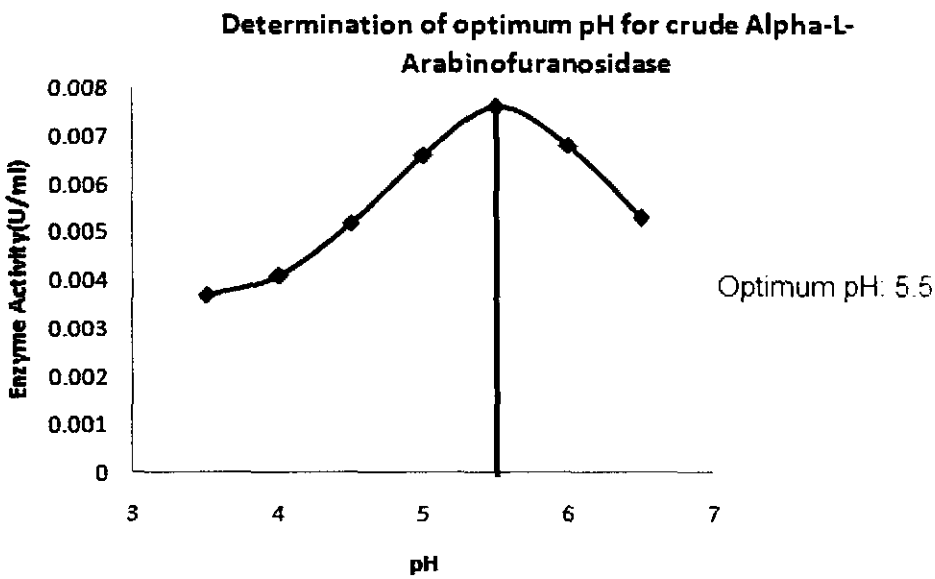
**Fig: 5.1** Staining of fungal *(Penicillium)* species

## 5.2 CHARACTERIZATION OF KINETIC PROPERTIES OF CRUDE α-L-ARABINOFURANOSIDASE

### 5.2.1. Effect of pH on crude α-L-arabinofuranosidase activity

The activity of crude α-L-Arabinofuranosidase activity was determined for different pH ranging from 3.5 to 6.5. The effect of pH on the enzyme activity has been shown in Fig 5.2. The enzyme was most active at pH 5.5 and less active at pH 3.5. The similar results obtained by other authors (Usama F.Ali, 2009, Hitomi, 2008, Mohamed *et.al.*, 2010). The result obtained reveals that the enzyme extracted was acid stable and it can used under acidic conditions. At a higher or lower pH than the optimum value, the glutamate residues (Glu 28, Glu 176, Glu 298) found in the catalytic site and Trp, Phe, Ile, Asn residues found in substrate binding site of the enzyme undergo deprotonation or protonation respectively, due to which a conformational change occurs in the active site of the enzyme. This alters the interaction between enzyme and substrate which results in the decreased activity of the enzyme (Cartmell, 2002).

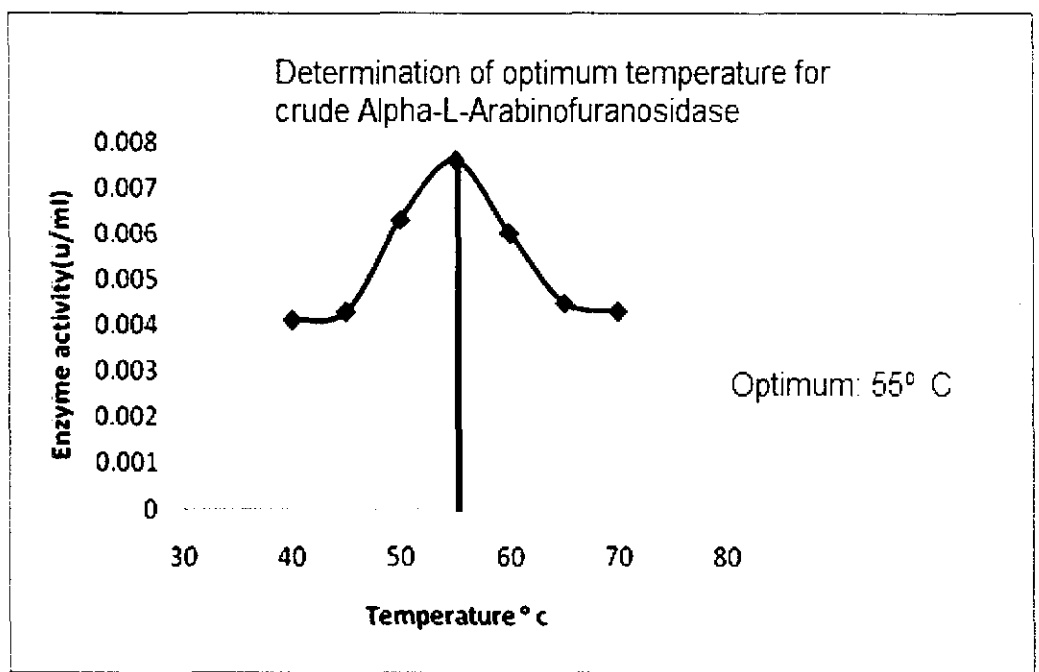
**Fig: 5.2 Determination of optimum pH for crude α-L-Arabinofuranosidase activity**



### 5.2.2 Effect of temperature on crude α-L-arabinofuranosidase activity

The enzyme activity was assayed at different temperatures ranging from 40° C to 70° C at pH 5.5. The effect of temperature on the enzyme activity is shown in Fig 5.3. The enzyme showed highest activity at 55° C and was less active at 40° C. Similar results were shown by other authors (Mohamed *et.al.*, 2010, Satoshi *et.al.*, 1998, Gindy *et.al.*, 2003). This showed that the enzyme was thermostable and it can be used in the high temperature conditions. At higher temperatures, the hydrogen bonds found in the enzyme undergo breakage due to which the enzyme gets denatured and structure gets distorted. So there will be a poor interaction between enzyme and substrate which results in the decreased activity of the enzyme.

**Fig: 5.3** Determination of optimum temperature for crude α-L-Arabinofuranosidase activity



### 5.2.3 Effect of Substrate concentration on the crude α-L-Arabinofuranosidase activity

The activity of crude α-L-Arabinofuranosidase was determined by varying the substrate concentration ranging from 20μM to 100μM. Michealis-Menten Plot was drawn the Km value for the substrate Paranitrophenyl α-L-Arabinofuranoside was found to be 25μM as shown in the Fig 5.4a and 5.4b. Then, an approximately similar value of 27μM was obtained from the Lineweaver Burk Plot as shown in the Fig 5.5. The enzyme extracted was found to possess significantly very low Km. whereas other researchers obtained a higher Km values (Tuncer, 2000, Badal, 1998). Since, only the crude enzyme was characterized, the results showed a low Km value. If the enzyme was purified, the Km value of the enzyme might increase or decrease which may comply the results obtained by different researchers.

**Fig: 5.4a** Michaelis-Menten Plot showing the effect of substrate concentration on the activity of crude α-L-Arabinofuranosidase

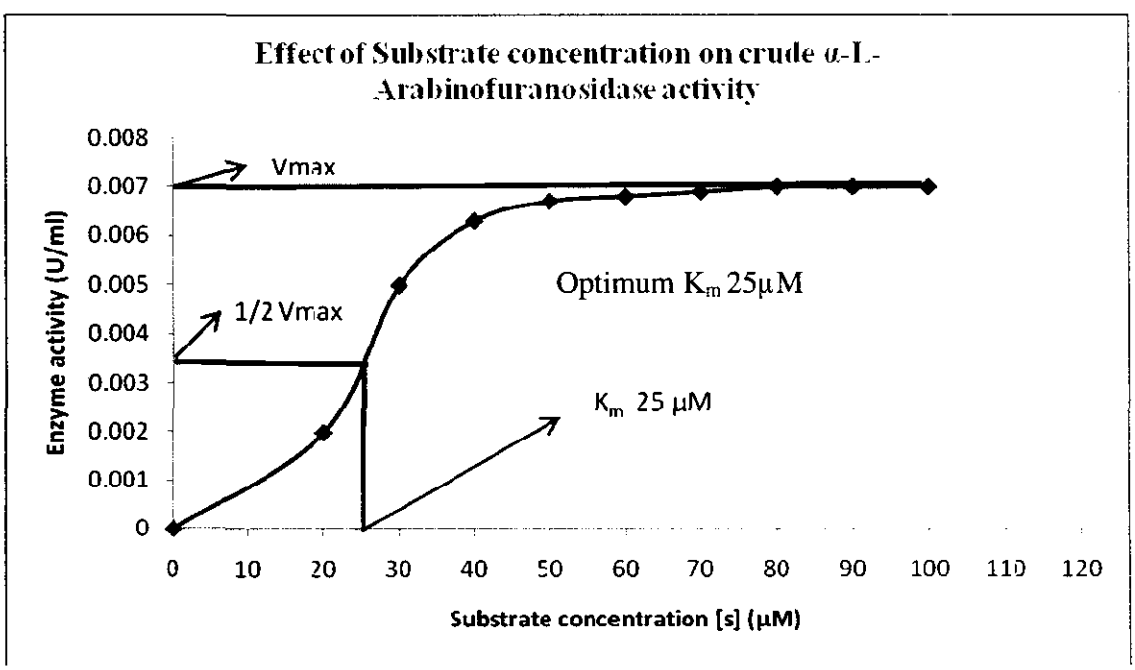


**Fig: 5.4b** Michaelis-Menten Plot showing the effect of substrate concentration on the activity of crude α-L-Arabinofuranosidase

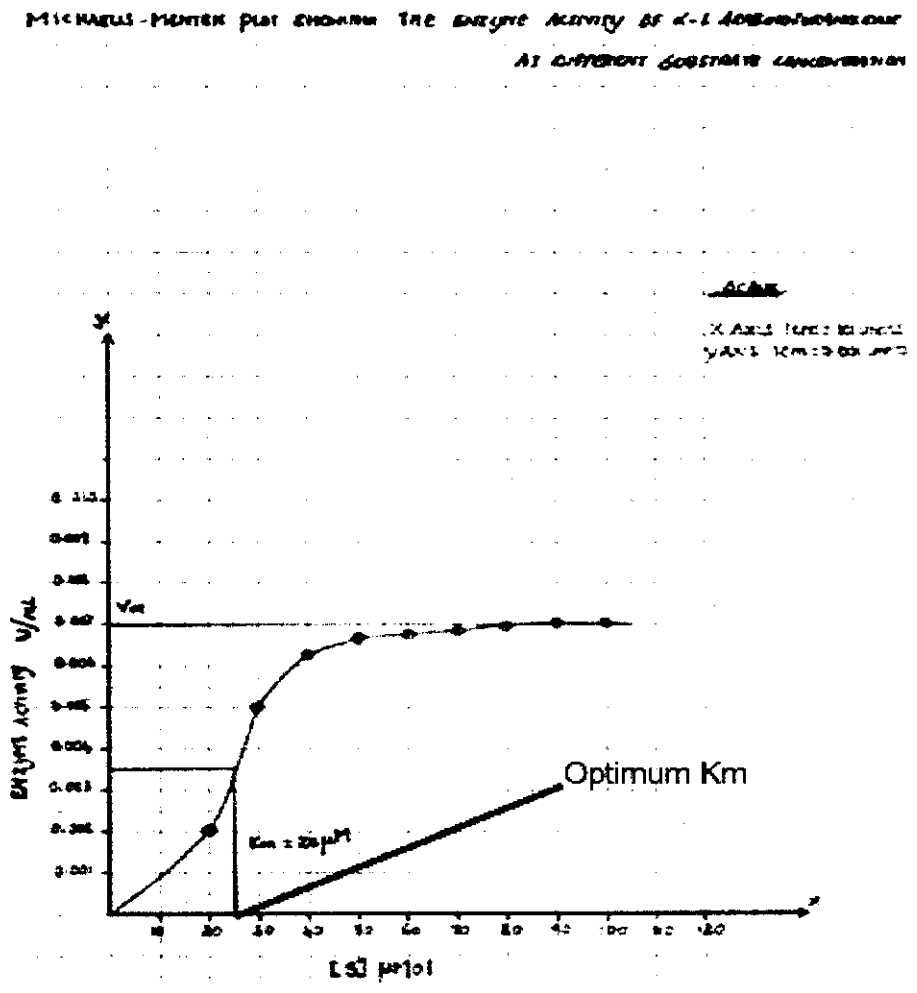
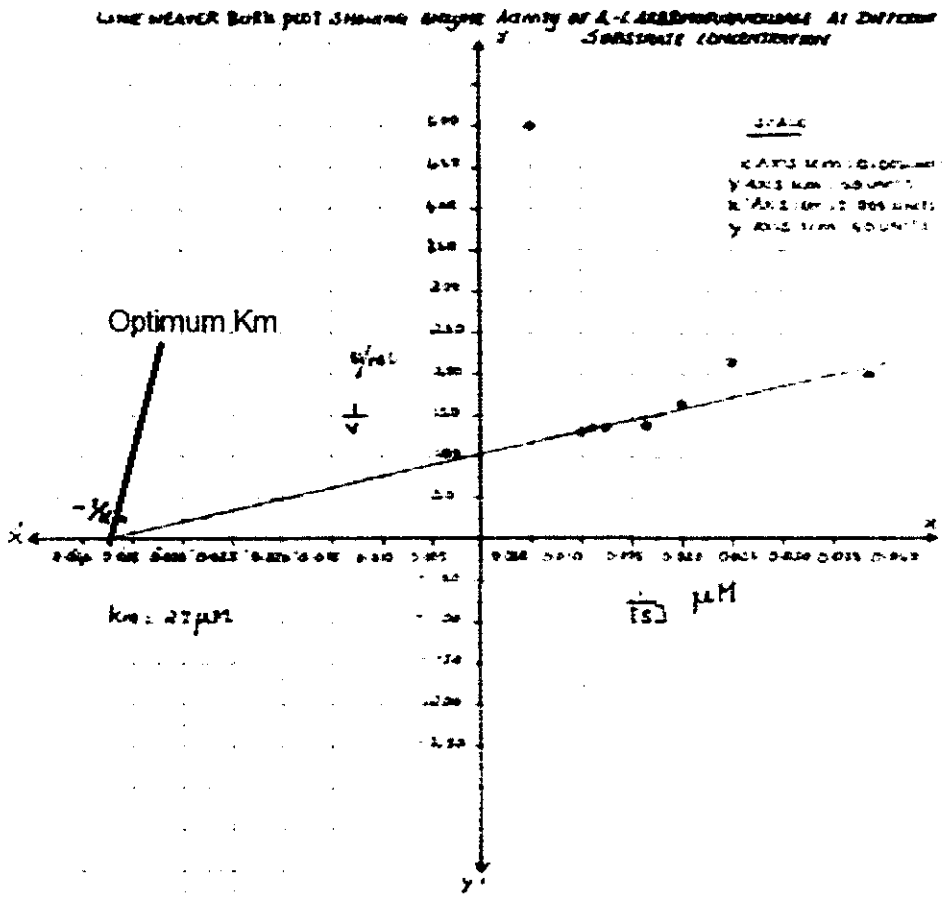


**Fig: 5.5** Lineweaver Burk Plot showing the effect of substrate concentration on the activity of crude α-L-Arabinofuranosidase



## 5.3 MEDIA OPTIMIZATION

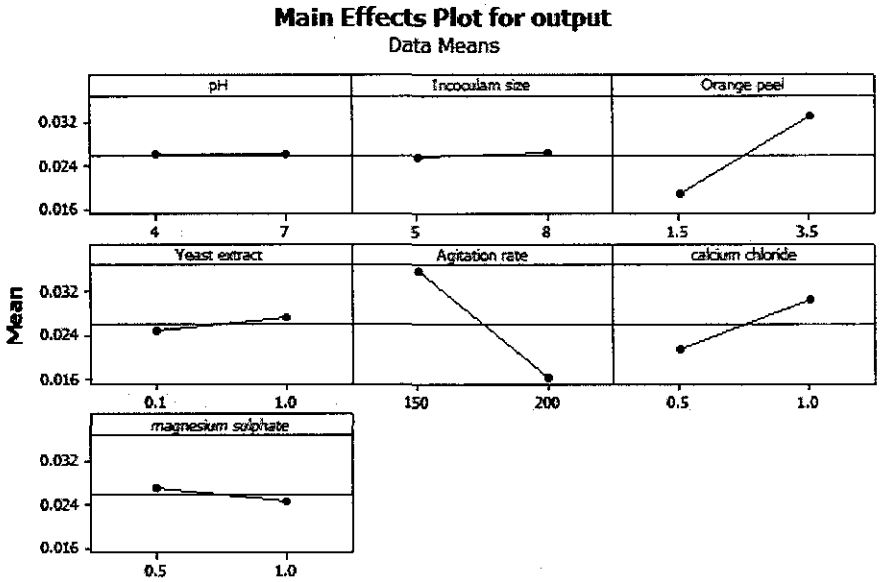
### 5.3.1. PLACKET BURMAN DESIGN

The Plackett–Burman design was used to investigate the effects of seven variables on α-L-Arabinofuranosidase production. The design matrix selected for screening the significant variables in α-L-Arabinofuranosidase production and their corresponding responses are given in (Table 5.1). Among the variables screened, Agitation rate (150 rpm), Orange peel (3.5%) and $CaCl_2$ (0.1 g/l) were identified as the most significant variables influencing α-L-Arabinofuranosidase production and the the maximum activity was observed as 0.045 U/ml. In fact, the Agitation rate value was noted to exert a negative effect on α-L-Arabinofuranosidase production (Agitation rate was inversely proportional to the enzyme activity). Orange peel and $CaCl_2$, however, were found to exert a positive effect. The values of all the other variables were statistically insignificant and were, therefore, not considered in the subsequent analysis. The effect of three significant variables on the activity of α-L-Arabinofuranosidase was described in the main effects plot as shown in the Fig 5.6. These significant variables may differ in other cases depending on the variables chosen (Mohamed *et.al.*, 2010).

**Table: 5.1** Combinations of seven variables and their effect on α-L-Arabinofuranosidase production.

| pH | Inoculum size(spores/loop) | Orange peel (%) | Yeast Extract(g/l) | Agitation rate(RPM) | $CaCl_2$ (g/l) | $MgSo_4$ (g/l) | Enzyme activity (U/ml) | |
|---|---|---|---|---|---|---|---|---|
| 4 | $10^8$ | 3.5 | 1.0 | 150 | 1.0 | 1.0 | 0.043 | |
| 4 | $10^5$ | 1.5 | 1.0 | 200 | 1.0 | 0.5 | 0.014 | |
| 7 | $10^8$ | 3.5 | 0.1 | 200 | 1.0 | 0.5 | 0.029 | |
| 4 | $10^8$ | 1.5 | 0.1 | 150 | 1.0 | 1.0 | 0.0364 | |
| 4 | $10^5$ | 3.5 | 1.0 | 200 | 0.5 | 1.0 | 0.019 | |
| 7 | $10^5$ | 3.5 | 1.0 | 150 | 1.0 | 0.5 | 0.0545 | Maximum activity |
| 4 | $10^8$ | 3.5 | 0.1 | 200 | 0.5 | 0.5 | 0.0189 | |
| 7 | $10^8$ | 1.5 | 1.0 | 200 | 0.5 | 1.0 | 0.0108 | |
| 7 | $10^8$ | 1.5 | 1.0 | 150 | 0.5 | 0.5 | 0.0209 | |
| 7 | $10^5$ | 3.5 | 0.1 | 150 | 0.5 | 1.0 | 0.034 | |
| 4 | $10^5$ | 1.5 | 0.1 | 150 | 0.5 | 0.5 | 0.025 | |
| 7 | $10^5$ | 1.5 | 0.1 | 200 | 1.0 | 1.0 | 0.006 | |

**Fig: 5.6** Main Effects Plot showing the effect of different variables on the crude α-L-Arabinofuranosidase activity




## 5.3.2 RESPONSE SURFACE METHODOLOGY

A Central Composite Design (CCD) was performed to determine the optimum levels of the significant factors (Orange peel percentage, Agitation rate and $CaCl_2$ concentration) and the effect of their interactions on α-L-Arabinofuranosidase production (Table 5.2). It can be noted that there was a considerable variation in the amount of α-L-arabinofuranosidase activity produced and that this variation depended heavily on the levels of the three independent variables (Orange peel percentage (2.5%), Agitation rate (132.95 rpm) and $CaCl_2$ concentration(0.75 g/l)) and the maximum activity obtained was 0.052 U/ml.The three-dimensional response surface and their corresponding contour plots were obtained on the basis of the model equation in order to determine the optimum level of each factor selected for maximum α-L-Arabinofuranosidase activity production by *Penicillium* species.

**Table: 5.2** Different combinations of three significant variables and their effect on α-L-Arabinofuranosidase production

| C5 | C6 | C7 | C8 |
|---|---|---|---|
| Agitation rate | Orange peel | CaCl2 | output |
| 132.955 | 2.50000 | 0.75000 | 0.052 |
| 175.000 | 2.50000 | 0.75000 | 0.017 |
| 150.000 | 1.50000 | 0.50000 | 0.027 |
| 200.000 | 1.50000 | 1.00000 | 0.016 |
| 175.000 | 2.50000 | 0.75000 | 0.015 |
| 175.000 | 2.50000 | 1.17045 | 0.024 |
| 175.000 | 2.50000 | 0.75000 | 0.019 |
| 200.000 | 3.50000 | 1.00000 | 0.019 |
| 150.000 | 3.50000 | 0.50000 | 0.036 |
| 150.000 | 3.50000 | 1.00000 | 0.041 |
| 175.000 | 2.50000 | 0.32955 | 0.016 |
| 217.045 | 2.50000 | 0.75000 | 0.010 |
| 175.000 | 2.50000 | 0.75000 | 0.016 |
| 175.000 | 4.18179 | 0.75000 | 0.027 |
| 175.000 | 2.50000 | 0.75000 | 0.014 |
| 150.000 | 1.50000 | 1.00000 | 0.039 |
| 200.000 | 3.50000 | 0.50000 | 0.017 |
| 175.000 | 0.81821 | 0.75000 | 0.011 |
| 200.000 | 1.50000 | 0.50000 | 0.014 |
| 175.000 | 2.50000 | 0.75000 | 0.012 |

Maximum activity

Fig 5.7a and 5.7b represents the 3D response surface plot and its corresponding contour plot respectively. It shows the effects of orange peel percentage and Agitation rate on α-L-Arabinofuranosidase production when $CaCl_2$ was fixed at its middle level (0.75 g/l). It can be noted that at a low Agitation rate,the increase in orange peel percentage induced a significant increase in α-L-Arabinofuranosidase production from 0.010 U/ml to 0.052 U/ml. At a higher Agitation rate, however, this increase was lower. These findings suggest that α-L-Arabinofuranosidase was better induced at lower Agitation rate and at high orange peel concentration.

Fig 5.7c represents the Low Response Contour Plot for the combined effects of Agitation rate and Orange peel percentage on the α-L-Arabinofuranosidase production. This plot depicts the ranges that should chosen while running the experiment because these ranges always lead to lower production of α-L-Arabinofuranosidase.

**Fig: 5.7a** Response Surface Plot for the combined effects of Agitation rate and Orange peel on the α-L-Arabinofuranosidase production

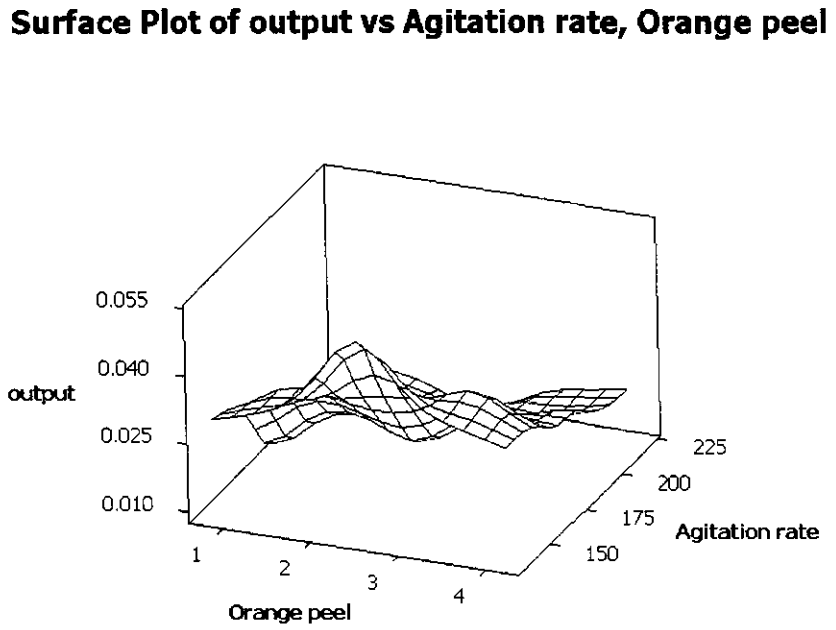



**Fig: 5.7b** Contour plot for the combined effects of Agitation rate and Orange peel on α-L-Arabinofuranosidase production

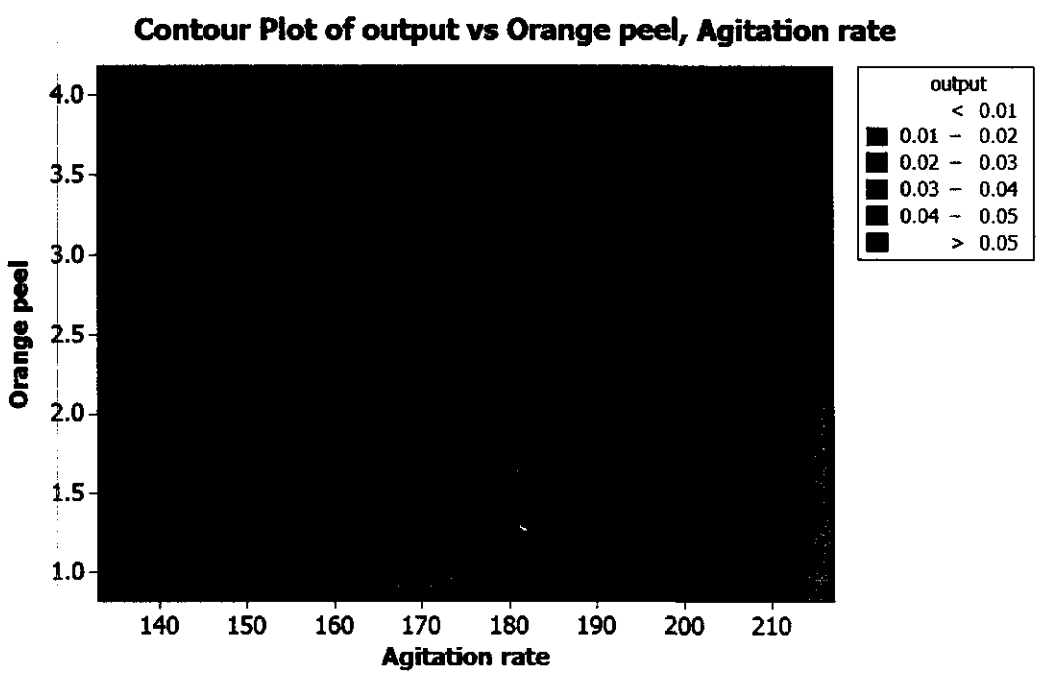


**Fig: 5.7c** Low Response Contour plot for the combined effects of Agitation rate and Orange peel on α-L-Arabinofuranosidase production

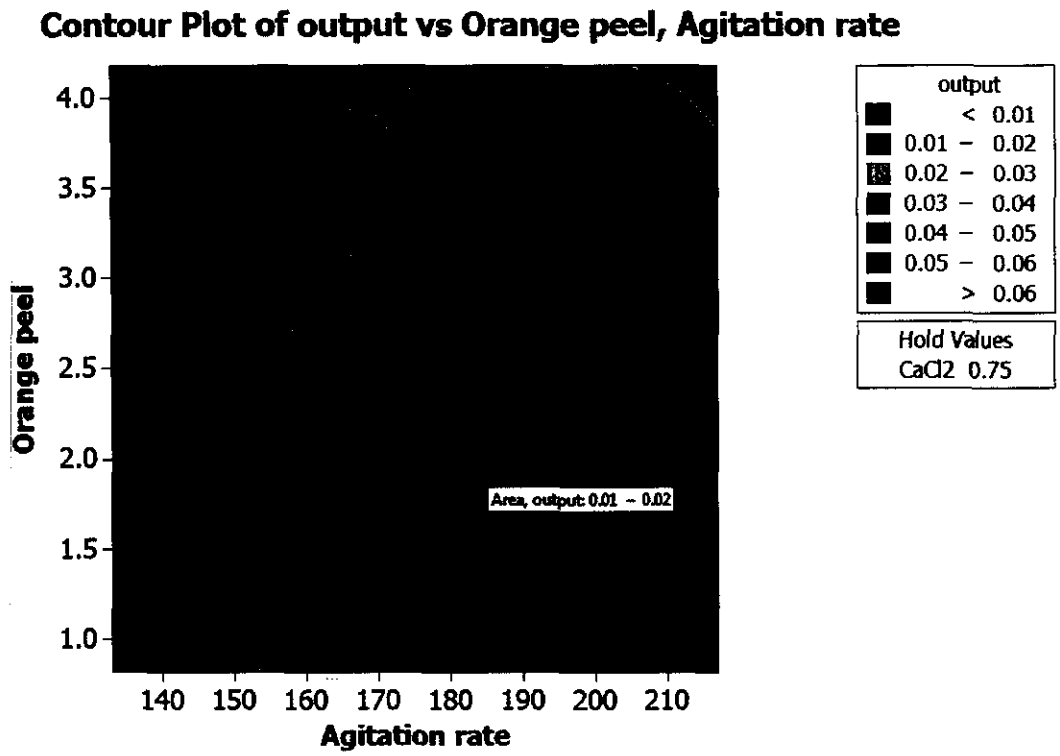



Fig 5.8a and 5.8b shows the 3D response surface plot and contour plot respectively. It shows the effects of Agitation rate and $CaCl_2$ concentration on α-L-Arabinofuranosidase production when orange peel percentage was fixed at its middle level (2.5%). When the Agitation rate of the culture broth was lower, the increase of $CaCl_2$ concentration favoured the accumulation of α-L-Arabinofuranosidase. However, no significant effect was observed for $CaCl_2$ on α-L-Arabinofuranosidase production at higher Agitation rate.

**Fig: 5.8a** Response Surface Plot for the combined effects of CaCl2 and Agitation rate on α-L-Arabinofuranosidase production

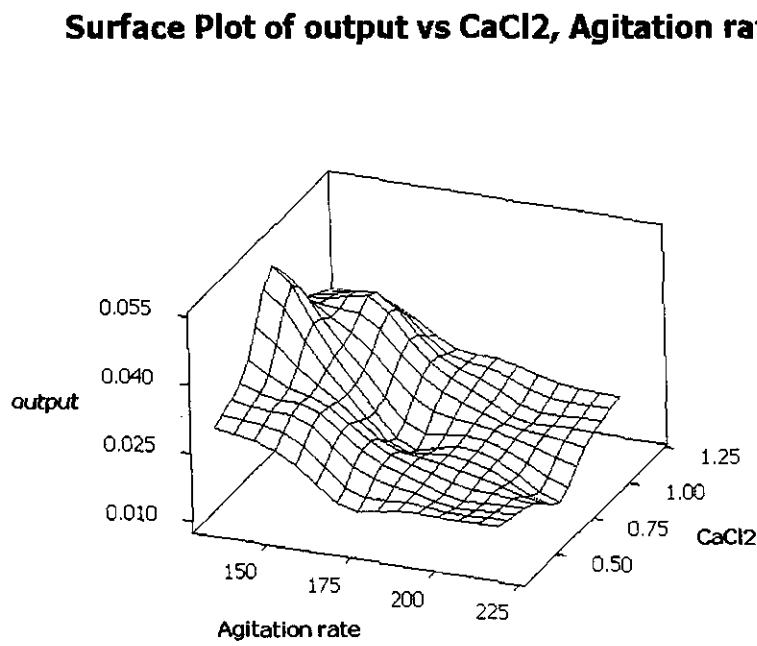
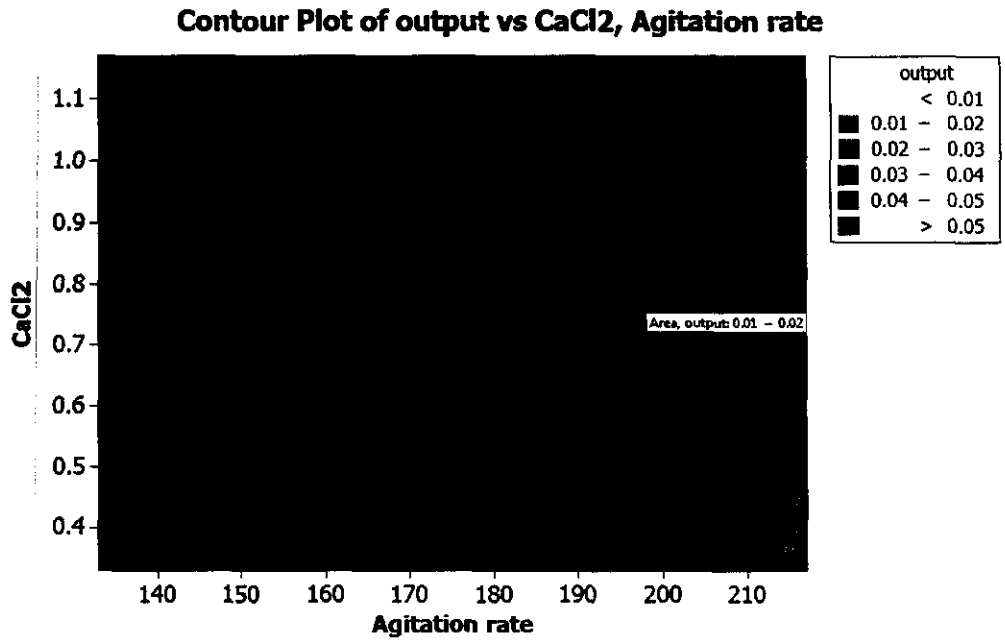



**Fig: 5.8b** *Contour Plot for the combined effects of* $CaCl_2$ *and Agitation rate on* α-L-Arabinofuranosidase production



These results correspond to the first trial media optimization experiments that gave us a very little higher response region. Further trials may be carried out to still optimize the media components and the plot with the greater area of higher response regions can be obtained.

## 5.4 INHIBITOR STUDIES

The effect of metal ions on the activity of crude α-L-Arabinofuranosidase was studied. The enzyme extract was assayed with the addition of different metal ions ($Al^{3+}$, $Zn^{2+}$, $Fe^{3+}$, $Ni^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Ca^{2+}$, EDTA, $Hg^{2+}$) each at a concentration of 1mM. The relative activity of the enzyme with the metal ions was found out by comparing its activity with the enzyme without the metal ion addition (Control- whose activity considered as 100%). The enzyme activity was highly inhibited by $Hg^{2+}$ (which showed 65% inhibition) and secondly by $Cu^{2+}$ (which showed 56% inhibition). $CaCl_2$ showed a very low inhibition (14% inhibition). Hence, along with the enhancers like arabinan, $CaCl_2$ may increase the activity of crude α-L-Arabinofuranosidase. The relative activity values for enzymes possessing different metal ions were given in the **Table 5.4.**

**Table: 5.4** Effect of metal ions on the crude α-L-Arabinofuranosidase activity

| S.NO | CONCENTRATION (1mM) | INHIBITORS | RELATIVE ACTIVITY (%) |
|---|---|---|---|
| 1 | 1 | $HgSO_4$ | 35 |
| 2 | 1 | $CuSO_4$ | 44 |
| 3 | 1 | $MnSO_4$ | 62 |
| 4 | 1 | $Al_2(SO_4)_3$ | 63 |
| 5 | 1 | $FeCl_2$ | 67 |
| 6 | 1 | EDTA | 69 |
| 7 | 1 | $ZnCl_2$ | 76 |
| 8 | 1 | $NiCl_2$ | 79 |
| 9 | 1 | $CaCl_2$ | 86 |

## 5.4 APPLICATIONS OF CRUDE α-L-ARABINOFURANOSIDASE

### 5.5.1FRUIT JUICE CLARIFICATION

**a) Apple juice clarification**

To check the application of crude α-L-Arabinofuranosidase in fruit juice clarification, the enzyme extract was added to the Apple juice and kept under incubation at room temperature for 4 days. Fig 5.9a and 5.9b shows the apple juice condition before and after clarification respectively. The figure clearly shows the colour change in the apple juice after clarification. The turbidity of fruit juice was also decreased after four days of incubation with enzyme extract. These results clearly explain the enzymatic treatment of fruit peel.

**b) Grape peel degradation**

Again to confirm the application of crude α-L-Arabinofuranosidase in fruit peel degradation, the crude enzyme extract was added to the grape peel of known dimension and kept under incubation at room temperature for 7 days. Fig 5.10a and 5.10b shows the grape peel at day1 after addition of enzyme extract and the grape peel at day 7 after addition of enzyme extract respectively. The figure apparently shows the change in the grape peel texture after 7 days of incubation with the enzyme extract comparatively with the unchanged grape peel texture in control. This proved the degradation of the grape peel by the action of crude α-L-Arabinofuranosidase.

Since only the crude enzyme was applied to the fruit juice and peel, probably, other enzymes like pectinases, xylanases would have contributed for the clarification and degradation. But the complete clarification was possible only in the presence of α-L-Arabinofuranosidase, the only enzyme that hydrolyzes the arabinofuranosyl side chains which are predominant in the hemicelluloses like arabinoxylan which is a great barrier in fruit juice clarification. The percentage of clarification and degradation can be calculated further measuring the viscosity and the turbidity of enzyme treated fruit juice and peel samples.

**Fig: 5.9a** Apple juice before clarification (Day-1)

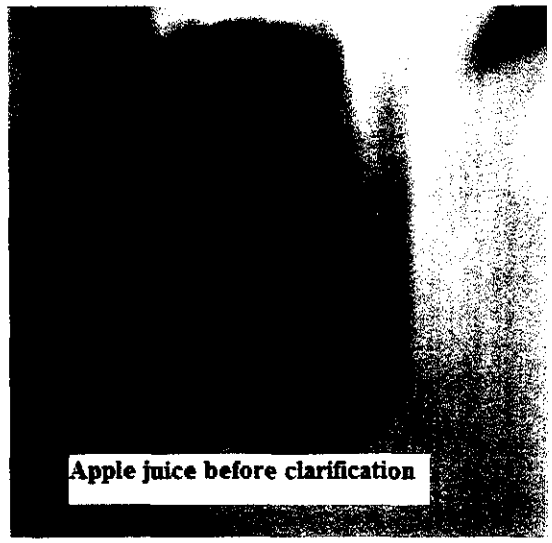



**Fig: 5.9b** Apple juice after clarification (Day- 4 after incubation)

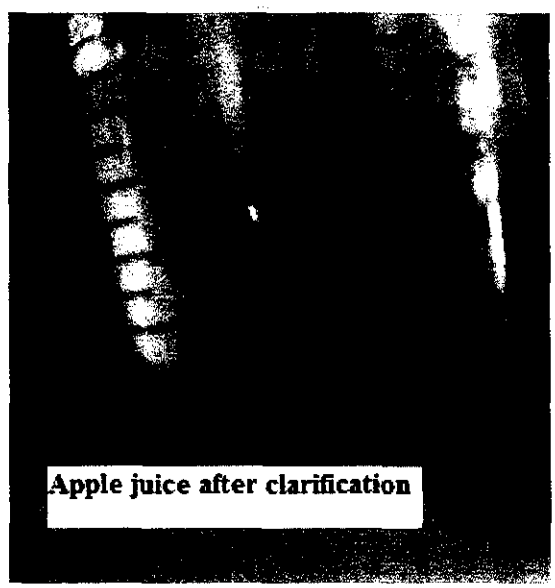


**Fig: 5.10a** Day-1 Grape peel condition after addition of enzyme extract

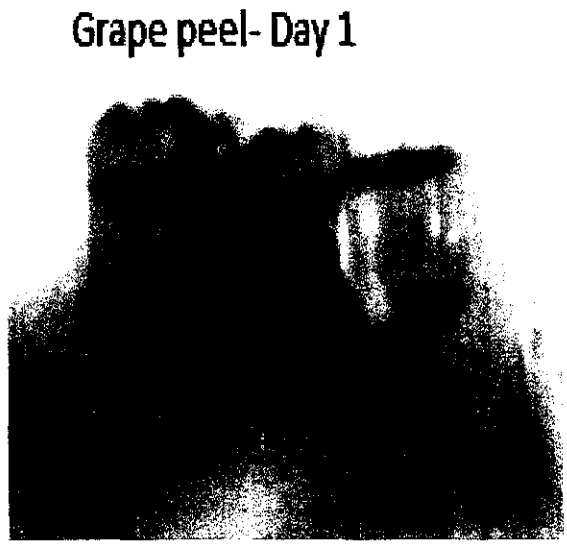
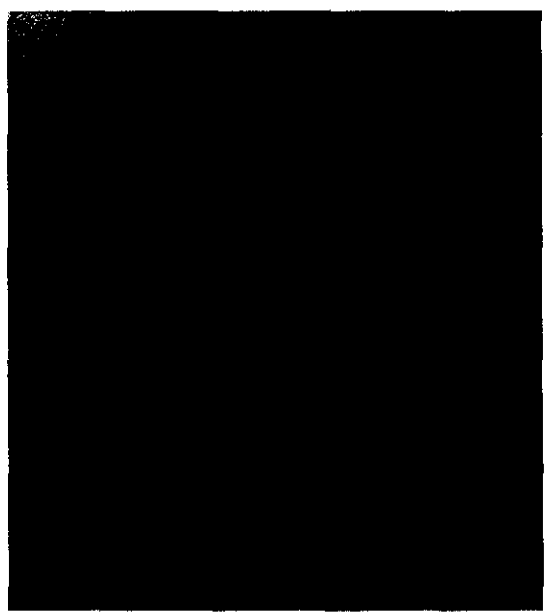


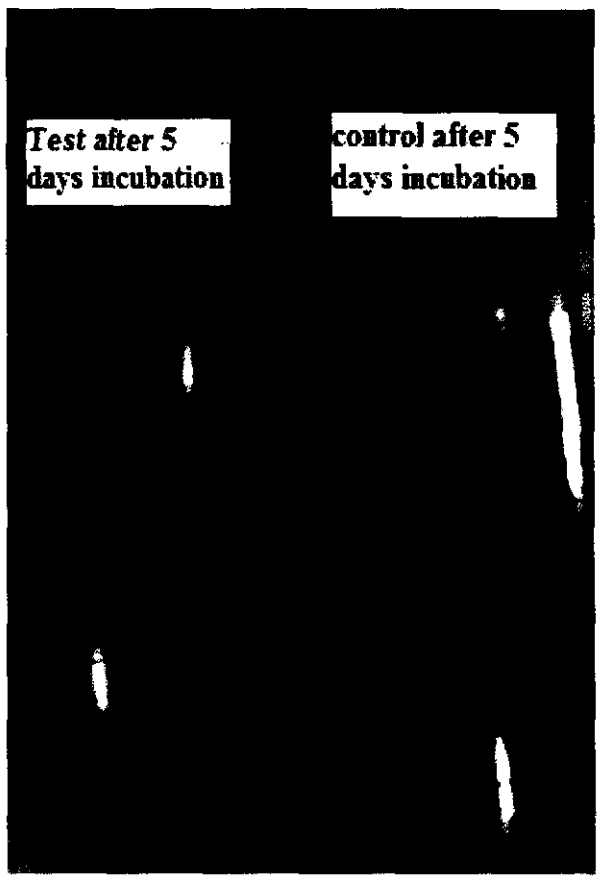

**Fig: 5.10b** Day-7 Grape peel condition after addition of enzyme extract



### 5.5.2. PAPER AND PULP DEGRADATION

Hearing that α-L-Arabinofuranosidase can be applied for paper and pulp degradation, the enzyme extract was added to the paper and pulp effluent sample and kept under incubation for 5 days at room temperature. Fig 5.11 shows the difference in the colour between control and test sample to which the enzyme extract was added. The turbidity of the pulp was also decreased after the incubation for 5 days when compared to the control which was obviously because of the enzymatic action on the pulp. Paper pulp contains higher amount of lignin. Since only crude enzyme was applied to the paper effluent, the degradation might be the combined effect of ligninases and side chain degrading enzymes like α-L-Arabinofuranosidases.

**Fig: 5.11** Effective paper and pulp degradation by the application of crude α-L-Arabinofuranosidase



# *Conclusion*

## 6. CONCLUSION

An attempt was made to identify and isolate α-L-Arabinofuranosidase producing organisms from the soil obtained from the foot hills of Anaikatti, Western Ghats, Coimbatore district. The colonies isolated as a pure culture was identified as *Penicillium* species and the activity of the crude enzyme was found to be 0.004 U/ml. The studies on kinetic properties of the crude α-L-Arabinofuraosidase has revealed an optimum pH of 5.5, temperature at 55°C and a low $K_m$ of 25μM. The Plackett Burman Design(PBD) screening has showed that out of seven selected various variables, Agitation rate (15o rpm), Orange peel (3.5%) and CaCl2 (0.1 g/l) were found to have significant effect in the production of α-L-Arabinofuranosidase and the maximum activity was observed as 0.045 U/ml. The Response Surface Methodology (RSM) analysis extended for the three selected variables confirmed that a combination of high concentration of Orange peel (2.5%), low Agitation Rate (132.95 rpm) and high concentration of $CaCl_2$ (0.75 g/l) provided a maximum enzyme activity at 0.052 U/ml. Different metals like 1mM $Hg^{2+}$ (65%),$Cu^{2+}$ (44%) showed different patterns of α-L-Arabinofuranosidase inhibitory activities. The crude α-L-Arabinofuranosidase enzyme was found to possess a significant apple juice clarification and paper pulp effluent degradation properties. From the overall analysis, it can be concluded that the present enzyme was acid and thermo stable and can be targeted for food industries with a commercial value.

# *References*

## 7. REFERENCES


1. Ali, U. F. (2009) 'Extracellular α-L-Arabinofuranosidase from *Aspergillus niger* and *A.oryzae'*, Aust J BasicAppl Sci., Vol. 3, No.3, pp.1984-1993.
2. Antoinette, C. O. (1997) 'Cellulose: the structure slowly unravels', cellul., Vol.4, pp.173-207.
3. Bhat, M.K. (2000) 'Cellulases and related enzymes in biotechnology', Biotechnol Adv., Vol.18, pp.355-383.
4. Christopher, L. Szakacs, G. Szendefy, J. (2006) 'Potential of solid-state fermentation enzymes of *Aspergillus oryzae* in biobleaching of paper pulp', Enzyme Microbiol Technol., Vol.39, pp.1354-1360.
5. Deloitte, 'Enhancing firm level competitiveness Indian food and agro processing industry' August, 2009.
6. El-Gindy, A.A. and Saad, R.R. (2003) 'An α-L-Arabinofuranosidase production by a thermophilic fungus *Thermomyces lanuginosus'*, Ann Microbiol., Vol.53, No.4, pp.437-445.
7. Fincher, G. B. Burton, R. A. Hrmova, M. and Lee, R. C. (2001) ' Barley arabinoxylan arabinofuranohydrolases : purification, characterization and determination of primary structures from cDNA clones', Biochem J.,Vol. 356, pp.181-189.
8. Fincher, G. B. Burton, R. A. and Gidley, J. M. (2010) 'Heterogeneity in the chemistry, structure and function of plant cell walls' Nat Chem Biol., Vol.6, pp.724-732.
9. Gobbetti M, Lavermicocca P, Minervini F, De Angelis M, Corsetti A (2000) 'Arabinose fermentation by *Lactobacillus plantarum* in sourdough with added pentosans and α-L-Arabinofuranosidase: a tool to increase the production of acetic acid' J Appl Microbiol., Vol.88, pp.317–324.
10. Gomes, E. R.D.S. Mendes, E. S. Pereira, N. C. Barros, S. T.D.D. (2005) 'influence at different operation conditions on the acerola juice concentration by reverse osmosis, using spiral membrane of composite film' Alim Nutr., Vol.16, No.4, pp.315-320.
11. Guerfali, M. Belghith,H. Chaabouni, M. and Gargouri, A. (2010) 'Improvement of alpha L-Arabinofuranosidase production by *Talaromyces thermophilus* and agro industrial residues saccharification', Appl Microbiol Biotechnol., Vol.85, pp.1361-1372.
12. Hoenich, N. (2006) 'Cellulose for medical applications' Bioresour., Vol.1, No.2, pp.270-280.

13. Ichinose, H. Fujimoto, Z. Kaneko, S. and Yoshida, M. (2008) 'Characterization of a modular enzyme of exo-1,5-α-L-arabinofuranosidase and arabinan binding module from *Streptomyces avermitilis* NBRC14893', Appl Microbiol Biotechnol., Vol.80, pp.399–408.
14. Kaneko, S. Demura, T. Ichinose, H. and Nishikubo, N. (2010) 'Characterization of a-L-arabinofuranosidase related to the secondary cell walls formation in *Arabidopsis thaliana*', Plant Biotechnol., Vol.27, pp.259–266.
15. Karangwa, E. Khizar, H. Rao, L. Nshimiyimana, D. S. Foh, M. B. K. Li, L. Xia, S. Q. and Zhang, X. M. (2010) 'Optimization of Processing Parameters for Clarification of Blended Carrot-orange Juice and Improvement of its Carotene Content' J Food Technol., Vol.2, No.5, pp.268-278.
16. Kimura, I. Sasahara, H. and Tajima, S. (1995) 'Purification and Characterization of Two Xylanases and an Arabinofuranosidase from *Aspergillus sojae'*, J Ferment Bioeng., Vol.80, No.4, pp.334-339.
17. Kusakabe, I. Sano, T. M. and Kaneko, S. (1994) 'Purification and Some Properties of α-L-Arabinofuranosidase from *Bacillus subtilis 3-6*', Appl Environ Microbiol., Vol.60, No.9, pp.3425-3428.
18. Kusakabe, I. Arimoto, M. Kaneko, S. Ohba, M. Kobayashi, H. and Ishii, T. (1998) 'Purification and Substrate Specificities of Two α-L-Arabinofuranosidases from *Aspergillus awamori* IFO 4033', Appl Environ Microbiol., Vol.64, No.10, pp.4021–4027.
19. Labavitch, J. M. Greve, L. C. and Hungate, R. E. (1984) 'α-L-Arabinofuranosidase from Ruminococcus albus 8: Purification and Possible Role in Hydrolysis of Alfalfa Cell Wall', Appl Environ Microbiol.,Vol.47, No.5, pp.1135-1140.
20. Laborda, F. Byrde, R. J. W. and Fielding, A. H. (1973) 'Extra- and Intra-cellular α- L-Arabinofuranosidase of *Sclerotinia fructigena'*, J Gen Microbiol., Vol.79, pp.321-329.
21. Li, L. K. Chen, C. T. Wan, C. F. and Huang, L. (2007) 'Expression, Purification and Characterization of a Bifunctional α-L-Arabinofuranosidase/β-D-xylosidase from *Trichoderma koningii* G-39', J Chin Chem Soc., Vol.54, pp.109-116.
22. MacCabe, P. A. and Polaina, J. (2007) 'Industrial enzymes: structure, function and applications' Springer, The Netherlands.

23. Martinez, G. A. Rosli, H. G. Civello, P. M. (2009) 'α-l-Arabinofuranosidase from strawberry fruit: Cloning of three cDNAs, characterization of their expression and analysis of enzymatic activity in cultivars with contrasting firmness', Plant Physiol Biochem., Vol.47, No.4, pp.272–281.
24. Mateo, J. J. Jimènez, M. (2000) 'Monoterpenes in grape juice and wines' J Chromatogr A. Vol.881, pp.557–567.
25. Millan, E. C. Chu, A. R. Escalante, J. A. M. Lopez, A. L. M. Reyes, C. M. B. (2009) 'Maize Arabinoxylan Gels as Protein Delivery Matrices' Molecules, Vol.14, pp.1475-1482.
26. Muhammad, A. K. (2010) 'Hydrolysis of hemicellulose by commercial enzyme mixtures', M.sc., Lulea University of Technology.
27. Nakas, J.P. and Makkonen, H.P. (2005) 'Use of xylanase and arabinofuranosidase for arabinose removal from unbleached kraft pulp', Biotechnol Lett., Vol. 27, pp.1675–1679.
28. Numan, M. T. and Bhosle, N. B. (2006) 'Alpha-L-Arabinofuranosidases: the potential applications in biotechnology', J Ind Microbiol Biotechnol., Vol.33, pp.247–260.
29. Pandey, A. and Nigam, P. S. (2000) 'Biotechnology for Agro-Industrial Residues Utilisation' Springer.
30. Pinphanichakarn, P. and Lauruengtana, V. (2006) 'Optimization of Culture Medium and Conditions for α-L-Arabinofuranosidase Production by *Streptomyces sp.* PC22', J Sci Res Chula Univ., Vol.31, No.1, pp.57-66.
31. Plackett, R. L. and Burman, J.P. (1946) 'The design of optimum multi factorial Experiments', Biometrika, Vol.33, pp.305–325.
32. Poutanen, K. and Puls, J. (1984) 'Characteristics of *Trichoderma reesei* β-xylosidase and its use in the hydrolysis of solubilized xylans', Appl Microbiol Biotechnol., Vol.28. No.5, pp.425-432.
33. Rangarajan, V. Rajasekharan, M. Ravichandran, R. Sriganesh, K. and Vaitheeswaran, V. (2010) 'Pectinase Production from Orange Peel Extract and Dried Orange Peel Solid as Substrates Using *Aspergillus Niger*', Int J Biotechnol Biochem., Vol.6, No.3, pp.445–453.
34. Roy, B. Wang, D. Park, E. Y. Yang, H. Jiang, L. Wan-fu, Y. Xing-hua, L. and Yun-gen, M. (2009) 'The most stirring technology in future: Cellulase enzyme and biomass utilization', Afr J Biotechnol., Vol.8, No.11, pp.2418-2422.

35. Saha, B. C. and Bothast, R. J. (1998) 'Purification and Characterization of a Novel Thermostable α-L-Arabinofuranosidase from a Color-Variant Strain of *Aureobasidium pullulans*', Appl Environ Microbiol., Vol.64, No.1, pp.216–220.
36. Saha, B. C. (2000) 'Alpha-L-Arabinofuranosidases: biochemistry, molecular biology and application in biotechnology', Biotechnol Adv., Vol.18, pp.403-423.
37. Saha, B. C. (2003) 'Hemicellulose bioconversion', J Ind Microbiol Biotechnol.,Vol.30, pp.279-291.
38. Salma, F. (2008) 'Investigation of β-xylosidase, α-L-arabinofuranosidase and acetylesterase from *Thermotoga hypogeal*', M.sc., University of Waterloo.
39. Sannino, A. Demitri, C. and Madaghiele, M. (2009) 'Biodegradable Cellulose-based Hydrogels: Design and Applications' 2009, Vol.2, pp.353-373.
40. Shin, H. Y. Bae, E. A. Choo, M. K. Park, S. Y. and Kim, D. H. (2002) 'Metabolism of ginsenoside Rc by human intestinal bacteria and its related antiallergic activity' Biol Pharm Bull, Vol.25, pp.743–747.
41. Shin H. Y. Park, S. Y. Sung, J. H. and Kim, D. H. (2003) 'Purification and characterization of α-L-arabinopyranosidase and α-L-arabinofuranosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb2 and Rc' Appl Environ Microbiol., Vol.69, pp.7116-7123.
42. Shoham, Y. and Gilead, S. (1995) 'Purification and Characterization of α-L-Arabinofuranosidase from *Bacillus stearothermophilus* T-6', Appl Environ Microbiol., Vol.61, No.1, pp.170–174.
43. Taylor, E. J. Smith, N. L. Turkenburg, J. P. D'Souza, S. Gilbert, H. J. and Davies, G. J. (2006) 'Structural insight into the ligand specificity of a thermostable family 51 arabinofuranosidase, Araf51, from Clostridium thermocellum' Biochem J., Vol.3953, No.1, pp.31-37.
44. Tuncer, M. (2000) 'Characterization of β-Xylosidase and α-L-Arabinofuranosidase activities from *Thermomonospora Fusca* BD25', Turk J Biol., Vol.24, pp.753–767.
45. Viikari, L. Kantelinen, A. Sundquist, J. and Linko, M. (1994) 'Xylanases in bleaching-from an idea to the industry, FEMS Microbiol Lett., Vol.13, pp.335-350.

46. Wagschal, K. Franqui-Espiet, D. Kibblewhite-Accinelli, E. R. Lee, C. C. Robertson, G. H. Wong, W. S. (2007) 'Genetic and biochemical characterization of an α-L-arabinofuranosidase isolated from a compost starter mixture' Enzyme Microb Technol., Vol.40, pp.747-753.