Chapter 1
CHAPTER 1

Introduction and Literatures Review

1.1 INTRODUCTION

Lignocelluloses of plant cell walls are composed of cellulose, hemicellulose, pectin and lignin (Kanderperker and Numan, 2008). Hemicellulloses are one of the most abundant renewable polymers on the earth. Moreover, cellulose, hemicelluloses, lignin and pectins are the key components in the degradation of lignocelluloses (Fig. 1.1). Many enzymes are involved in the degradation of these polymeric substrates (Ward and Moo-Young, 1989). L-Arabinosyl residues are widely distributed in these polymers as side chains. The presence of these side chains restricts the enzymatic hydrolysis of hemicelluloses and pectins (Rahman et al., 2003; Saha, 2000; Saha and Bothast 1998a,b). Further, it also represents a formidable technological barrier that retards the development of various industrial processes (Saha, 2000). The use of a single accessory enzyme for partial or specific modification of lignocelluloses might offer new interesting options for the utilization of these low-cost raw materials (Leathers, 2003; Sknchez-Torres et al., 1996). The α-L-arabinofuranosidases (α-L-AFases) are accessory enzymes that cleave α-L-arabinofuranosidic linkages and act synergistically with other hemicellulases and pectic enzymes for the complete hydrolysis of hemicelluloses and pectins (Margolles-Clark et al., 1996; Spagna et al., 1998). These enzymes warrant substantial research efforts because they represent potential rate-limiting enzymes in the degradation of lignocelluloses from agricultural residues (Saha, 2000). The action of α-L-AFase alone or in combination with other lignocellulose-degrading
enzymes represents a promising biotechnological tool as alternatives to some of the existing chemical technologies such as chlorination in pulp and paper industry (Gobbetti et al., 1999; Gomes et al., 2000; Mai et al., 2000), synthesis of oligosaccharides (Rémont et al., 2002; Rémont et al., 2004) and pretreatment of lignocelluloses for bioethanol production (Saha and Bothast, 1998b; Saha, 2003). Considering the potential and future prospects of α-L-AFases, this literatures review deals with the various aspects of these enzymes with emphasis on their potential for biotechnology.

![Cellulose bundler](Image)

**Fig. 1.1**: The complex composition and structure of lignocelluloses. Modified from Khandeparker and Numan (2008).

### 1.2 Plant cell wall polysaccharides

The chemical and physical structure of the lignocelluloses plant cell wall varies depending on the plant group and cell type. However, there are some general characteristics that are common to all plant cell walls. Plant cell walls are not chemically homogeneous and contain distinct layers of several different substances. Cell walls contain microfibrillar polysaccharides (cellulose) and matrix polysaccharides, which are divided into hemicelluloses and pectins (Goodwin and Mercer, 1983). Other components present in plant cell wall are
lignin, proteins, encrusting substances (cutin, suberin, inorganic compounds), and water (Aspinall, 1980; Goodwin and Mercer, 1983). The general structure of lignocelluloses is presented in Figure 1.1. Cellulose is the most abundant polysaccharides on the earth. It consist of unbranched chains of β-1,4-linked D-glucopyranose residues which are thousands of units long. Celllobiose is the repeating unit in the cellulose molecule. Cellulose polymers are usually arranged to form crystalline microfibils interrupted by regions with relatively low crystallinity (amorphous or paracrystalline cellulose) (Aspinall, 1980; Goodwin and Mercer, 1983). Hemicelluloses and pectins are the matrix polysaccharides of the plant cell wall. They account for 25–35% of lignocellulose biomass (Saha, 2000).

1.2.1 Hemicelluloses

Hemicelluloses are the second most abundant polysaccharides in nature after cellulose. They are low-molecular weight polysaccharides which are closely associated with cellulose and lignin. Hemicelluloses have a heterogeneous composition of various sugar units. Their primary structure depends on the type of the plant which can vary among different members of plant group or even from different tissues or wall layers of the same plant (Timell, 1967; Aspinall, 1980; Goodwin and Mercer, 1983). Hemicelluloses could be divided into xylans, mannans, 1,3 and 1,4-β-D-glucans and galactans. Xylans contain a β-1,4-linked D-xylose backbone (De Vries and Visser, 2001). In many plants, xylan backbone is substituted by different side chains with L-arabinose, D-galactose, acetyl, feruloyl, p-coumaroyl and glucuronic residues (Adams et al., 2004; De Vries and Visser, 2001). Xylans from grasses, cereals, softwood and hardwood differ in their composition. This is due to the differences in the
frequency and composition of the side chain substituents of xylans. Thus, xylans could be found in different forms such as arabinoxylans and arabinoglucuronoxylans (Saha, 2000; De Vries and Visser, 2001; Saha, 2003).

1.2.1.1 Arabinoxylans

Arabinose-containing xylans (or arabinoxylan) are hemicellulosic structures found mainly as the secondary wall components in gymnosperms and monocotyledonous (Aspinall, 1980). Arabinoxylans constitute the major fraction of cereal cell wall polysaccharides. The starchy endosperm cell walls of cereal grains are mainly composed of arabinoxylans (60-70%) except in the case of barley (~20%) and rice (27-40%) (Fincher and Stone, 1986). Similarly, arabinoxylans are found in the cell walls of the grasses belonging to the family Gramineae (Kroon and Williamson, 1996; Adams et al., 2004). Arabinoxylans contain xylan backbone that is partially substituted at intervals with α-L-arabinofuranose residues (Adams et al., 2004). Moreover, wheat arabinoxylan also contains other substituents as shown in Figure 1.2 (De Vries and Visser, 2001; Adams et al., 2004). The manner of attachment of arabinose units to the xylan backbone has been a matter of continuous research. The linkages of Arabinofuranose (Araf) to C(0)3 and to C(O)2,3 of xylose residues have been reported (Ebringerova et al., 1990). More recently, the presence of another linkage type, namely Araf linked to C(O)2 of Xylopyranose (Xylp) residues, has been verified for arabinoxylans (Izydorczyk and Biloaderis, 1995). A small proportion of oligomeric side-chains consisting of two or more arabinosyl residues linked via 1→2, 1→3 and 1→5 linkages has been reported although most arabinofuranosyl residues in arabinoxylans are found as monomeric substituents. Although arabinoxylans from various cereals and/or
various plant tissues share the same basic chemical structure, they also differ in some additional aspects. These included differences in the ratio of arabinose to xylose, in the relative proportions and sequence of the various linkages between these two sugars and in the presence of other substituents (Izydorczyk and Bilideris, 1995). Ferulic acid and acetyl substituents are found at intervals on cereal arabinoxylans and they are known to modify to some extent the solubility, and the interaction between the arabinoxylans and solvents (Fincher and Stone, 1986).

![Diagram of arabinoxylan structure](image)

**Fig. 1.2:** The main structural features considered present within water-soluble wheat arabinoxylans. The diagram also indicates the variety of enzymes that are active against arabinoxylans. Modified from Adams *et al.* (2004).

The distribution of substituents along the 1,4-β-D-xylan backbone is not even and several models for cereal arabinoxylans have been proposed (Gruppen *et al.*, 1993; Saulnier and Thibault, 1999). In highly branched regions single- and double- substituted residues are clustered together and are interrupted by less
substituted or unsubstituted regions. It is also possible that two structurally distinct polymers exist. Molecular weight values reported for cereal arabinoxylans varied extensively depending on the fraction, source of the sample and the analysis method. Molecular weights ranging from about $2 \times 10^4$ to $1.7 \times 10^6$ were reported for cereal arabinoxylans (Fincher and Stone, 1986). Softwoods contain 10-15% arabin-4-O-methylglucuronoxylan. The backbone is substituted by 1,3-linked $\alpha$-L-arabinofuranose and 1,2-linked 4-O-methyl-$\alpha$-D-glucouronic acid residues with ratios to xylopyranose residues of 1:8-9 and 1:5-6, respectively (Timell, 1967). In comparison to the other softwood arabinoglucuronoxylans, the majority of the arabinofuranose side groups in larchwood arabinoxylan are attached to the C-2 of the xylopyranose residues instead of the C-3. In addition, a minor amount of double substituted xylopyranoses exists (Kormelink and Voragen, 1993). Birch wood (Roth) xylan contains 89.3% xylose, 1% arabinose, 1.4% glucose, and 8.3% anhydrousuronic acid (Kormelink and Voragen, 1993). Rice bran neutral xylan contains 46% xylose, 44.9% arabinose, 6.1% galactose, 1.9% glucose, and 1.1% anhydrousuronic acid (Shibuya and Iwasaki, 1985). Wheat arabinoxylan contains 65.8% xylose, 33.5% arabinose, 0.1% mannose, 0.1% galactose, and 0.3% glucose (Gruppen et al., 1992). Corn fiber hemicellulose contains 48–54% xylose, 33–35% arabinose, 5–11% galactose and 3–6% glucuronic acid (Doner and Hicks, 1997; Hespell, 1998; Saha, 2000).

1.2.1.2 Pectins

Pectins are important cell wall matrix polysaccharides which have a key role in mechanical strength and adhesion between cells (Aspinall, 1980). They are a family of complex heteropolysaccharides that contain two well-defined regions
called as smooth and hairy (Fig. 1.3) (Catoire et al., 1998; De Vries and Visser, 2001). The three pectic polysaccharides homogalacturonan, rhamnogalacturonan-I and substituted galacturonan have been isolated from plant cell walls (De Vries et al., 1982; De Vries and Visser, 2001). The dominant feature of the pectins is the presence of a linear backbone of galacturonic acid containing varying proportion of methyl ester groups.

![Diagram of pectin structure](image)

**Fig. 1.3:** Schematic drawing of pectin (rhamnogalacturonan I) showing the smooth and hairy regions. Modified from De Vries and Visser (2001).

Pectin polymer backbone is interspersed at intervals with rhamnose residues carrying the neutral sugars side chains containing arabinose and galactose that form arabinans, arabinogalactans or galactans (Fig. 1.3) (Catoire et al., 1998; Habibi et al., 2004). Pectins are abundant in the soft tissues of citrus fruits (about 30%) (Whitaker, 1984; Habibi et al., 2004), sugar beet pulp (25%), apple (15%) (De Vries et al., 1982; Churms et al., 1983) and other land plants. Woody tissues contain only minor amounts of pectins (Aspinall, 1970;
Goodwin and Mercer, 1983). Pectins could be divided into polyuronic acids, arabinans, and galactans.

1.2.1.2a Arabinans

Arabinans are pectic homopolysaccharides that are highly branched and consist predominantly of α-L-arabinofuranose residues (Tagawa and Kaji, 1988). Arabinan consists of a main chain of α-1,5-linked L-arabinose residues that can be substituted by α-1,3-linked L-arabinose and feruloyl residues attached terminally to O-2 of the arabinose residues (Fig. 1.4a).

a) Branched arabinan

\[
\longrightarrow 5)-\alpha-L-Araf-(1\rightarrow 5)-\alpha-L-Araf-(1\rightarrow 5)-\alpha-L-Araf-(1\rightarrow 5)-\alpha-L-Araf-(1\rightarrow 5)-\alpha-L-Araf-(1\rightarrow 3)
\]

\[\uparrow \quad \uparrow \quad \uparrow \]

\[1 \quad 1 \quad 1\]

\[\alpha-L-Araf \quad \alpha-L-Araf \quad \alpha-L-Araf\]

b) Debranched arabinan

\[
\longrightarrow 5)-\alpha-L-Araf-(1\rightarrow 5)-\alpha-L-Araf-(1\rightarrow 5)-\alpha-L-Araf-(1\rightarrow 5)-\alpha-L-Araf-(1\rightarrow 5)-\alpha-L-Araf-(1\rightarrow 3)
\]

Fig. 1.4: Primary structure of a) Arabinan and b) Debranched Arabinan. Adopted from Rahman et al. (2003).

Arabinan also can be decorated at C-2 and/or C-3 with arabinofuranose molecules as side chains (Aspinall, 1980; Beldman et al., 1993; Ralet et al., 1994; Beldman et al., 1997). These side chains restrict the enzymic hydrolysis of respected polysaccharide (Rahman et al., 2003). Removing the side chains of arabinan result in the formation of a linear or debranched arabinan (Fig 1.4b).

Arabinans have been isolated from sugar beet, peanut, apple, citrus pectins, carrot, cabagge, rape and mustard seeds. Arabinans have been found also in the

1.2.1.2b Arabinoglactans

Depending upon the substitutions attached to the backbone arabinoglactans can be classified into three types. These include arabino-1,4-β-D-galactan, arabino-1,3/6-β-D-galactan and a related but a distinct group of cell wall glycoproteins containing arabinose and galactose. Arabinogalactan backbone is composed mainly of 1,3-linked D-galactopyranose units (Eriksson *et al.*, 1990). In all cases however, arabinogalactan backbone is usually substituted mainly at C-6 by single L-arabinose and/or D-galactose residues or longer branched arabinan or arabinogalacto-oligomers. Furthermore, considerable structural differences have been found between different members of the same arabinogalactan type (Clarke *et al.*, 1979; Aspinall, 1980).

Arabino-1,4-β-D-galactans are present as pectic complexes in seeds, bulbs and leaves, and coniferous compression wood (van de Vis, 1994). Whereas, arabino-1,3/6-β-D-galactans are found in mosses, coniferous woods, gums, saps and exudates of angiosperms, seeds, leaves, roots and fruits of higher plant, and in suspension of cultured plant cells. Furthermore, these arabinogalactans are also constituents of many exudate gums of angiosperms (e.g. *Acasia*) and of gymnosperms (e.g. genus *Larix*), and of pectic complexes (Timell, 1967; Clarke *et al.*, 1979; van de Vis, 1994).

1.3 Degradation of hemicelluloses and pectins

Hemicelluloses and pectins are complex heteropolysaccharides, and a vast variety of synergistically acting main-chain and side-chain cleaving enzymes
are needed for their complete hydrolysis. Several enzymes hydrolysing hemicelluloses and pectins have been identified and characterized from both bacterial and fungal sources.

Xylans are the most common hemicelluloses and the enzyme systems needed for their degradation are relatively well known. Endo-1,4-β-D-xylanases (EC 3.2.1.8) randomly attacking the backbone of β-1,4-linked xylans, have been isolated from bacteria, fungi and even plants (Coughlan and Hazlewood, 1993; Coughlan and Hazlewood, 1993; Kulkarni et al., 1999). Xylo-oligosaccharides formed by the xylanases are further degraded by exo-1,4-β-D-xylosidases (EC 3.2.1.37), which remove D-xylose residues from the non reducing end of the substrate. Their activity normally decreases with increasing depolymerisation of the substrates (Coughlan and Hazlewood, 1993).

Depending on the origin of xylan, different side-group cleaving enzymes are needed for complete degradation of the substrate (Fig.1.2). Arabinose side-group in arabinoxylans of softwood and annual plants are removed by α-L-arabinofuranosidases. The α-D-Glucuronidases (EC 3.2.1.131) hydrolyze the α-1,2-glycosidic linkage between D-glucuronic acid or its 4-O-methyl ether and the main-chain xylose residue (Poutanen et al., 1991). In addition, esterases (EC 3.1.1) librating the acetic and phenolic components are required for the complete hydrolysis of xylans from hardwood and annual plants (Christov and Prior, 1993).

The breakdown of neutral components of pectic polysaccharides, i.e. arabinans and arabinogalactans, requires the action of several enzymes. Endo-1,5-α-L-arabinanase (E.C.3.2.1.99) degrade the backbones of both linear and branched arabinan. However, the action of these enzymes can be limited by the high
degree of arabinose substitutions of the substrate. The bacterial endo-
arabinanases produce arabinose and arabinobiose as end products, whereas
arabinobiose and arabinotriose are accumulated in the reaction mixture when
fungal enzymes are used. Exo-α-L-arabinanases probably attack the
α-1,5-linked side-chains of beet arabinan exo-wise, releasing predominantly
oligosaccharides with three arabinose residues. However, linear arabinan is not
degraded by these enzymes. However, α-L-arabinofuranosidases (E.C.3.2.1.55),
which are discussed in greater details in the following sections, release single L-
arabinose units from both arabinans and arabinogalactans (McCleary, 1991;
Beldman et al., 1993; Beldman et al., 1997; Saha, 2000; Numan and Bhosle,
2006).

1.4 The α-L-arabinofuranosidases (α-L-AFases)
The α-L-AFases (α-L-arabinofuranoside arabinofuranohydrolases, EC 3.2.1.55)
are the enzymes involved in the hydrolysis of L-arabinose linkages. These
enzymes have been purified from several bacteria, fungi and plants (Hashimoto
and Nakata, 2003; Lee et al., 2003; Rahman et al., 2003). They form a part of
the array of glycoside hydrolases required for the complete degradation of
arabinose-containing polysaccharides (Saha, 2000; Takao et al., 2002). The
action of these enzymes accelerates the hydrolysis of the glycosidic bonds by
more than 10^{17} fold, making them one of the most efficient catalysts known
(Rye and Withers, 2000; Shallom et al., 2002). Such enzymatic hydrolysis
releases soluble substrates, which are utilized by both prokaryotic and
eukaryotic microorganisms (Margolles-Clark et al., 1996). The α-L-AFases
specifically catalyze the hydrolysis of terminal nonreducing-α-L-1,2-, α-L-1,3-
and α-L-1,5-arabinofuranosyl residues from different oligosaccharides and polysaccharides (Saha and Bothast, 1998b; Saha 2000; Sozzi et al., 2002). Whereas, the nature of a glycone sugar can influence the catalytic activity of other arabinose-releasing enzymes, the α-L-AFases do not distinguish between the saccharide link to the arabinofuranosyl moiety and thus exhibit wide substrate specificity (Romboust et al., 1988; Rahman et al., 2003). Effective hydrolysis of α-L-arabinofuranosyl residues from various pectic, homohemicellulosic polysaccharides (branched arabinans, debranched arabinans), heteropolysaccharides (arabinogalactans, arabinoxylans, arabinoxylloglucans, glucuronoxarabinoxylans, etc.) and different glycoconjugates is carried out by the α-L-AFases (Beldman et al., 1997; Sozzi et al., 2002). Moreover, most microbial α-L-AFases are secreted into the culture media; thus, they are likely to attack polysaccharides (Matuso et al., 2000).

1.4.1 The synergistic role of α-L-AFases

The importance of α-L-AFases has come from the fact that arabinose side chains on hemicelluloses and pectins participate in cross-linking within the plant cell wall structure. The presence of these side chains also affects the form and functional properties of hemicelluloses and pectins (De Vries et al., 2000). They reduce the interaction between polymers chains due to their inherently more flexible water-hungry furanose conformations. Moreover, the L-arabinofuranoside substitutions on xylan strongly inhibit the action of xylan-degrading enzymes (Fig. 1.2), thus preventing the complete degradation of the polymer to its basic xylose units (Saha 2000; Shallom et al., 2002). Similarly, L-arabinofuranoside substitutions in pectin (Fig. 1.3) prevent the complete
degradation of this polymer to its basic units. The α-L-AFases act synergistically with other hemicellulases and pectinases for the complete degradation of hemicelluloses and pectins, respectively (Bachmann and McCarthy 1991; Kormelink and Voragen, 1993; De Vries et al., 2000; Sakamoto and Kawasaki, 2003). Moreover, in some cases, α-L-AFases possessing β-xylosidase activity or xylanases with α-L-arabinofuranosidase activity also have been described (Utt et al., 1991; Matte and Forsberg, 1992; Mai et al., 2000; Lee et al., 2003). Furthermore, some α-L-AFases with both exo- and endo-activity on arabinan, one of the major constituents of pectins, has been reported (Birgisson et al., 2004; Miyazaki, 2005). The role of α-L-AFases in the degradation of arabinose-containing polymers is well known. They have a cooperative role facilitating the action of other lignocellulose-degrading enzymes (Tuncer, 2000; Tuncer and Ball, 2003a). This has been confirmed for α-L-AFase from Thermomonospora fusca that worked in truly synergistic relationship with endoxylanase from the same bacterium releasing 0.6 and 0.3 mg of reducing sugars from oat spelt xylan and ballmilled wheat straw, respectively (Bachmann and McCarthy, 1991). α-L-AFase played an important role to increase the release of reducing sugars from these lignocelluloses. However, other authors report the synergistic action of these enzymes with other pectinases and hemicellulases on lignocelluloses. For instance, the two enzymes α-L-AFases (kabfA and kabjB) from Aspergillus kawachii acted synergistically with xylanase in the degradation of arabinoxylan, releasing higher amounts of ferulic acid in the presence of feruloyl esterase (Koseki et al., 2003). Furthermore, Hashimoto and Nakata (2003) showed that hemicellulose from soy sauce materials was decomposed synergistically by xylanase,
β-xylosidase and α-L-AFase produced by *Aspergillus oryzae* HL15 during moromi fermentation. They also suggested that α-L-AFase of *A. oryzae* HL15 was very closely involved in releasing not only arabinose but also xylose into moromi mash. The same effect has been shown when these enzymes act synergistically on arabinoxylan. Moreover, an exo-arabinanase, Abnx from *Penicillium chrysogenum*, released very little arabinobiose from arabinan, as the action of Abnx was inhibited by the arabinofuranose unit linked as a side chain (Sakamoto and Kawasaki, 2003). When Abnx acted in combination with either α-L-AFases (AFQ1 or AFS1), from the same fungus, the arabinose contents in the reaction mixtures were higher than the sum of those by the two enzymes acting separately (Sakamoto and Kawasaki, 2003). Furthermore, Morales *et al.* (1999) reported that the two α-L-AFases, i.e., AF64 and AF53 from *Bacillus polymyxa*, facilitate the action of the endoxylanase on oat spelt xylan and wheat bran arabinoxylan. An increase in the production of smaller xylooligosaccharides has occurred because of the cooperative action of α-L-AFases used in these experiments (Morales *et al.*, 1999). α-L-AFases also act synergistically with endo-arabinanase and cinnamoyl esterase (CinnAE) from *Aspergillus niger*. When sugar-beet pulp (SBP) was incubated with the mixture of the former enzymes, the esterase was able to release 14 times more of the alkali-extractable ferulic acid present in the whole pulp as free acid than CinnAE alone (Kroon and Williamson, 1996).

1.4.2 Bifunctional α-L-arabinofuranosidase

A bifunctional enzyme is an enzyme containing two distinct catalytic capacities in the same polypeptide chain. They usually catalyze two consecutive reactions
Moreover, bifunctional enzymes usually catalyze complex multi-substrate reactions whose mechanisms involve a large number of intermediate enzyme forms. These features potentially allow manifestation of some new specific properties of bifunctional enzymes. First, the possible mobility of an intermediate (a product of the first reaction and at the same time, substrate of the second reaction) between two active sites without its appearance in solution (Meek et al., 1985; Miles et al., 1999; Huang et al., 2001). Second, the state of the active site of the first reaction may influence kinetic properties of the active site of the second reaction, and vice versa, the state of the active site of the second reaction may influence kinetic properties of the active site of the first reaction (Liang and Anderson, 1998).

Bifunctional (or polyfunctional) enzymes seem to appear in evolution by the combination of genes encoding enzymes tightly bound functionally (Yourno et al., 1970; Smith, 1994). At the protein level, the association of individual enzymes in the bifunctional or multifunctional enzyme complexes provides several distinct advantages. Catalytic events that take place on one enzyme can have a direct influence on the associated enzymes that are present in the complex. Single regulatory sites or regulatory subunits can control the coordinated activities of all of the enzymes in the complex. In addition, the directed transfer of reactants from consecutive active sites can support more efficient metabolism. Enzymes that catalyze sequential reactions tend to evolve toward bi or multifunctional enzymes for more efficient metabolism (Seo et al., 2000). This evolution may also occur under circumstances where the substrates of microbial enzymes are proximate, e.g., in rumen microorganisms capable of
exploiting plant fibers (Gosalbes et al., 1991; Gilbert et al., 1992; Xue et al., 1992; Flint et al., 1993).

Xylan degradation is a multistep process involving multiple enzymatic activities. Xylanases are extracellular enzymes that hydrolyze the internal $\beta$-1,4-xylosidic linkages of the xylan backbone structure. Xylanase action is restricted by the presence of side chains. Removal of side-chain substituents requires additional enzymatic activities of arabinofuranosidase, $\beta$-xylosidase uronidase, glucosidase, mannosidase, and acetyl esterase (Numan and Bhosle, 2006). The xylanase gene xysA of *Streptomyces halstedii* JM8 was used to isolate a DNA fragment from a gene library of the lignocellulolytic actinomycete *Streptomyces chattanoogensis* CECT-3336. Nucleotide sequence analysis revealed a gene (x1n23) encoding a bifunctional multimodular enzyme bearing two independent xylanase and $\alpha$-L-arabinofuranosidase domains separated by a Ser/Gly-rich linker. The N terminus of the predicted protein showed high homology to family F xylanases. The C terminus was homologous to amino acid sequences found in enzymes included in the glycosyl hydrolase family 62 and, in particular, to those of $\alpha$-L-arabinofuranosidase from *Streptomyces lividans*. PCR and RT-PCR experiments showed that the nucleotide sequences corresponding to each domain are arranged on the chromosomal DNA and they are co-transcribed (Hernandez et al., 2001). To our knowledge, this is the only report that described xylanase and arabinofuranosidase domains in the same open reading frame (Khandeparker and Numan, 2008). Furthermore, several fungal and a few bacterial $\beta$-xylosidases/$\alpha$-arabinosidases have been purified and characterized, often followed by cloning and analysis of respective gene. Most of these enzymes exhibit a high degree of substrate specificity; however, a
few enzymes, including the bifunctional xylosidase-arabinofuranosidase from *Thermoanaerobacter ethanolicus*, exhibit the highest substrate affinity towards the arylxylosides, but also the highest activity with arylarabinosides (Shao and Wiegel, 1992). Common to these enzymes is substrate inhibition by *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX). Several hemicellulases from different anaerobes, primarily ruminant bacteria, have been reported to have both β-xylosidase and α-L-arabinosidase activities encoded by a single gene product. Domains bearing different xylanolytic activities have been described previously for the xylanase (XynC) from *Fibrobacter succinogenes* (Manelius *et al.*, 1994; Zhu *et al.*, 1994; Mai *et al.*, 2000). A bifunctional protein (xylB) with xylosidase and arabinofuranosidase activities from *Bifidobacterium fibriiosolvens* has been reported (Ult *et al.*, 1991). In *Bacteroides ovatus*, (xsA), α-L-arabinofuranosidase and β-xylosidase activities were suggested to be catalyzed by a bifunctional protein of very similar weight (Whitehead and Hespell, 1990). Gasparic *et al.* (1995a) isolated a (xynB) gene from *Prevotella ruminicola β14* containing activities against *p*NPAF and *p*NP-xyloside. In addition to the above enzymes, *Clostridium stercorarium* xylA (Sakka *et al.*, 1993) have a bifunctional xylosidase and arabinofuranosidase activities and all of these enzymes contained numerous regions of sequence identity (Gasparic *et al.*, 1995a; Mai *et al.*, 2000). *Trichoderma koningii* arabinofuranosidase/β-xylosidase which belongs to the family 54 of glycosyl hydrolases has a catalytic activity that hydrolyse the terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides and 1,4-β-D-xylans so as to remove successive D-xylose residues from the non-reducing termini (CAZY, http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). *Butyrivibrio fibrisolvens* xylB β-xylosidase /
β-xylosidase / α-N-arabinofuranosidase has a 1.6-fold higher activity as an arabinosidase than as a α-xylosidase when tested on the substrates nitrophenyl-β-D-xylopyranoside and pNPA. Its catalytic activity is the hydrolysis of 1,4-β-D-xylans so as to remove successive D-xylose residues from the non-reducing termini as well as hydrolysis of terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides. This enzyme belongs to family 43 of glycosyl hydrolases (Ult et al., 1991). *Streptomyces lividans* α-L-arabinofuranosidase abfB liberates arabinose from arabinooligosaccharides and, after prolonged incubation, abfB exhibits some xylanolytic activity as well (CAZY, http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). The bifunctional enzymes xylosidases/α-arabinosidases may show differences in their activity as α-arabinosidases or xylosidases. The bifunctional xylosidase–arabinosidase (xarB) from thermophilic anaerobe *Thermoanaerobacter ethanolicus* JW200 showed different temperature optima for α-arabinosidases (65–85°C) and for xylosidase 88°C (Mai et al., 2000). These differences in temperature ranges for the xylosidase and the arabinosidase activities could be due to either (1) the presence of separate domains for each activity or (2) strong interactions with different side groups of amino acids in the catalytic center, which includes the possibility of different conformational changes of the enzyme (Mai et al., 2000).

1.4.3 Regulation of biosynthesis of microbial α-L-AFases

The regulation mechanism of the biosynthesis of α-L-AFases is well studied in fungi grown under Submerged Fermentation (SmF) (Rombouts et al., 1988; Witteveen et al., 1989; Gueimonde et al., 2007). In *Aspergillus niger* and *Penicillium chrysogenum* induction of α-L-AFases is under the control of at
least two regulatory systems: one by the pathway-specific induction by pentose sugars and polyols generated through the L-arabinose and D-xylose catabolism while the other by carbon catabolite repression by glucose (van der Veen et al., 1993; 1994). There are few reports on the regulation mechanism of the biosynthesis of α-L-AFases by bacteria. The pathway of L-arabinose utilization in *Bacillus subtilis* has been described first by Lepesant and Dedonder (1967). After transporting by permease and entering the cell, L-arabinose is sequentially converted to L-ribulose, L-ribulose 5-phosphate, and D-xylulose 5-phosphate by the action of L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively. D-Xylulose-5-phosphate is further catabolized through the pentose phosphate pathway (Sá-Nogueira and Lencastre, 1989). At this point D-xylose is also transported to the cell by AraE permease and enters the pathway after conversion to D-xylulose and D-Xylulose 5-phosphate by D-xylose isomerase and D-xylulose kinase, respectively (Gartner et al., 1988).

The *B. subtilis* genes involved in the utilization of L-arabinose (ara genes) characterized so far are those belonging to the metabolic araABDLMNPQ-abfA operon (Sá-Nogueira et al., 1997; Kunst et al., 1997), and the divergently arranged AraE/AraR genes encoding the main transporter for L-arabinose and a regulatory protein, respectively (Kunst et al., 1997; Sá-Nogueira and Mota, 1997; Sá-Nogueira and Ramos, 1997; Raposo et al., 2004). The abfA present in ara operon is the gene coding for α-L-AFase (Sá-Nogueira and Ramos, 1997).

The molecular mechanism of the regulation of gene expression by AraR, suggests that the regulatory protein acts as repressor by binding directly to DNA. The interaction of this catabolite repressor protein with DNA inhibit by the presence of the effector. L-Arabinose (the effector) acts as a typical inducer
that inhibits binding of the repressor protein to its control sites. The model proposed for the action of AraR is that, in the absence of L-arabinose, AraR binds to operator site(s) within the araABDLMNQP-abfA operon promoter and within the AraE/AraR promoters region, thus preventing transcription of the ara genes. The presence of L-arabinose induces a conformational change in AraR such that recognition and binding to DNA is no longer possible, thus allowing expression of the ara genes including abfA (Sá-Nogueira and Mota, 1997; Sá-Nogueira and Ramos, 1997; Raposo et al., 2004). In other hand, in *B. subtilis* AraE permease is the main transporter for L-arabinose (Sá-Nogueira and Ramos, 1997, Raposo et al., 2004) and responsible for the transport of the pentose D-xylose into the cell (Krispin and Allmansberg, 1998). Thus, utilization of L-arabinose and D-xylose by this bacterium is subjected to AraR regulation. Raposo et al. (2004) suggested that the expression of genes encoding extracellular degrading enzymes of arabinose-containing polysaccharides, transport systems, and intracellular enzymes involved in further catabolism is regulated by a coordinate mechanism triggered by arabinose via AraR. Furthermore, Mota et al. (1999) suggested that AraR may also control other genes and operons and may represent a global regulator of carbohydrate metabolism. However, other studies suggested that induction of the *B. subtilis* pentose (arabinose and xylose) catabolic operons, as well as global carbon catabolite repression, is mediated by several transcriptional repressors (Hueck and Hillen, 1995; Brückner and Titgemeyer, 2002).

The phenomenon of catabolite repression of the synthesis of α-L-AFases in the presence of readily metabolizable substrates such as glucose was indicated in some bacteria and fungi. These included *Butyrivibrio fibrisolvens* GS113.
(Hespell and O'Bryan, 1992); *Butyrivibrio fibrisolvens* H17c (Hespell and Cotta, 1995); *Thermotoga thermarum* (Sunna and Antranikian, 1996); *Bifidobacterium adolescentis* DSM (Van Laere et al., 1999); *Bifidobacterium longum* (Gueimonde et al., 2007); *P. ruminicola βl4* (Gasparic et al., 1995a); *Aspergillus niger* (Ruijter et al., 1997); *Aspergillus nidulans* (Gielkens et al., 1999) and *Penicillium purpurogenum* (De Ioannes et al., 2000; Carvallo et al., 2003). Nevertheless, all these studies were conducted by growing bacteria and fungi under SmF. However, studies indicated the importance of the growth environment on the expression of the catabolic enzymes (Te Biesebeke et al., 2002; 2005). Furthermore, growth environment in Solid State Fermentation (SSF) greatly differ than that for SmF. Furthermore, molecular biological studies showed that different proteins are produced and genes differentially transcribed in SSF vs. SmF (Te Biesebeke et al., 2002). Moreover, Te Biesebeke et al. (2005) suggested that different control mechanisms regulate the transcription of genes coded for hydrolytic enzymes in microorganisms grown under SSF and SmF. Hence, the regulation of biosynthesis of α-L-AFase by catabolite repression may also be different when the bacterium is grown under SSF. However, studies concerning the effect of catabolite repression on biosynthesis of bacterial α-L-AFase in SSF are not available.

1.4.4 Microbial production of α-L-AFases

The α-L-AFases production is influenced by the carbon source and composition of the growth medium. Various carbon sources including monomeric sugars and complex polysaccharides have been used to assess their effect on the production and induction of α-L-AFases. For example, pentoses D-arabinose, L-arabinose,
D-xylose and hexoses D-galactose, D-glucose, D-mannose, L-sorbose have been commonly used. Other sugars cellulbiose, lactose, lactulose, maltose, mellibiose, sucrose, trisaccharide, raffinose, D-arabitol, L-arabinol, D-mannitol, D-sorbitol and xylitol also have been used. Sugar beet pulp (starch-free), wheat bran (starch-free), wheat straw, oatmeal, rice straw and corn cob are some of the lignocelluloses that have been used for the production of α-L-AFases. Polysaccharides such as oat spelt xylan, birchwood xylan, beechwood xylan, wheat arabinoxylan, arabinogalactan, larch wood arabinogalactan, sugar beet arabinan, galactan CMC, guar gum, gum Arabic and locust bean gum have also been used. Pectins, schizophyllan, starch, xanthan, carboxymethyl cellulose, potato β-1,4-galactan, carob galactomannan, Me-β-xylloside and lactobionic acid are some other carbon sources utilized for α-L-AFases production. Generally, arabinose-containing substrates are essential for the efficient production of α-L-AFases (Beylot et al., 2001a;b; Koseki et al., 2003). Monomeric compounds L-arabitol and L-arabinose induce the genes involved in the production of these enzymes in some microorganisms (De Ioannes et al., 2000). Conversely, other monosaccharides such as glucose and galactose may inhibit the production of α-L-AFases (Beylot et al., 2001a;b; Koseki et al., 2003). Arabinogalactans and oatmeal were found to be the best inducers for α-L-AFase isolated from Bacillus pumilus PS213 (Degrassi et al., 2003). Rhodothermus marinus produced α-L-AFase when grown on birchwood xylan (Gomes et al., 2000). L-Arabitol induced production of α-L-AFases enzymes araA and araB by the A. niger mutants (de Groot et al., 2003); ABF1 by the Penicillium purpurogenum (De Ioannes et al., 2000; Carvallo et al., 2003) and kabfA and kabjB by the A. kawachii (Koseki et al., 2003). However, the
production of α-L-AFase by Pseudomona cellulosa was repressed when glucose was used in the growth medium (Beylot et al., 2001a). The high levels of enzyme production (1.0 U/ml) were obtained when arabitol was used as a carbon source for growth of Penicillium purpurogenum, while 0.85 and 0.7 U/ml are produced with sugar beet pulp and oat spelt xylan, respectively (De loannes et al., 2000). L-Sorbose, an excellent inducer of cellulase and xylanase from Trichoderma reesei PC-3-7, also induced AF activity (Nogawa et al., 1999). Both arabinoxylan arabinofuranohydrolases (AXH-d3 and AXH-m23) from Bifidobacterium adolescentis were induced when grown on xylose and arabinoxylan-derived oligosaccharides (Van Laere et al., 1999). In the case of Aureobasidium pullulans, arabinose was most effective for production of both whole-broth and extracellular α-L-AFase, followed by arabitol. However, oat spelt xylan, sugar beet arabinan, xylose, xylitol, and wheat arabinoylan were intermediate in their ability to support the α-L-AFase production (Saha and Bothast, 1998a). In the presence of arabitol, Aspergillus terreus CECT 2663 produced three α-L-AFases (Le Clinche et al., 1997). The filamentous fungus Cochliobolus carbonum produced α-L-AFase when grown on maize cell walls (Ransom and Walton, 1997). Roche and Durand (1996) studied the fungal Thermoascus aurantiacus solubilization of cell wall components of sugar beet pulp during solid-state fermentation. The α-L-AFase was found to be one of the highest enzyme activities present in the growth medium of this fungus. Bacillus stearothermophilus T-6 produced an α-L-AFase when grown in the presence of arabinose, sugar beet arabinan, or oat spelt xylan (Gilead and Shoham, 1995). Pretreated corn cob was the best substrate for production of extracellular α-L-AFase (4.2 U/ml) by
Lachke, 1995). Formation of α-L-AFase was induced in T. reesei RUT C-30 by growing the fungus on arabinose or dulcitol, and by adding arabinose, arabitol, galactose, or dulcitol to nongrowing mycelia (Kristufek et al., 1994). The maximum α-L-AFase production by Thermoascus aurantiacus was obtained by solid-state fermentation of the fungus on a leached sugar beet pulp-based medium (Roche et al., 1994). The highest levels of α-L-AFase were generated when the culture of A. nidulans was grown on 1% (w/v) purified beet pulp arabinan (Fernández-Espinar et al., 1994). Wheat bran was the best inducer for the production of α-L-AFase by Streptomyces diastaticus (Tajana et al., 1992). Arabinan, as the carbon source, was the most effective substrate for the production of α-L-AFase by Corticium rolfsii (Kaji and Yoshihara, 1970). Arabinan-containing carbon sources have efficiently induced α-L-AFase production in A. nidulans (Ramón et al., 1993), A. niger (van der Veen et al., 1991), various strains of Streptomyces (Kaji et al., 1981; Higashi et al., 1983; Johnson et al., 1988) and the phytopathogenic fungus Sclerotinia sclerotiorum (Riou et al., 1991).

The experiments carried out by Gomes et al. (2000) indicated that carbon and nitrogen sources influence the production of α-L-AFase by Rodothermus marinus. In these experiments, different concentrations of xylan (2–6 g/L) and yeast extract (4–12 g/L) were used to increase the enzyme production. The highest enzyme activity (108 nkat/ml) was obtained with the medium containing 3 and 9 g/L of birchwood xylan and yeast extracts, respectively. The lowest enzyme activity (86 nkat/ml) was obtained with medium containing 5 and 7 g/L of birchwood xylan and yeast extracts, respectively (Gomes et al., 2000). A. niger showed highest α-L-AFase activity (243 U/ml) when grown on a SSF
medium with C: N ratio of 15:9. The carbon and nitrogen sources used were dried skins of grape pomace and casein peptone, respectively (Huerta-Ochoa et al., 2003). Thermomonospora fusca BD25 showed highest α-L-AFase activity (0.136 U/mg protein) when grown in a medium containing 0.6% (w/v) oat spelt xylan and 0.6% (w/v) yeast extract corresponding to C:N ratio of 4:1 (Tuncer et al., 1999; Tuncer and Ball, 2003a,b). Production of α-L-AFase by A. nidulans was high when ammonium sulfate or ammonium chloride was used as nitrogen source (Fernández-Espinar et al., 1994). Aspergillus kuwachii IF04308 produced highest amounts of α-L-AFase when grown on a medium supplemented with a mixture of bactotryptone, yeast extract and NaNO₃ as a nitrogen source (Koseki et al., 2003). Similarly, when a mixture of urea, ammonium sulfate and neopeptone was used as a source of nitrogen, the production of α-L-AFase by Penicillium purpurogenum was enhanced (De Ioannes et al., 2000). The optimal medium composition for α-L-AFase production in solid-state fermentation by T. aurantiacus was sugar beet pulp containing 77.8% moisture (after being wetted with a mineral solution at pH 9.5) supplemented with 1.2% yeast extract as the nitrogen source (Roche et al., 1994).

Both temperature and pH of the growth medium are known to influence growth and enzyme production by microorganisms. The thermophilic bacterium R. marinus produced 5.32 U/mg of α-L-AFase when grown in a shake flask for 96 h at 61°C and pH 8 (Gomes et al., 2000). Similarly, the fungus Penicillium chrysogenum 31B produced higher amounts of two α-L-AFases when grown under static conditions for 12 days at pH 5.0 and 30°C (Sakamoto and Thibault, 2001; Sakamoto and Kawasaki, 2003).
Batch cultivation system in shake flasks has been used for α-L-AFases production by bacteria (Gomes et al., 2000; Degrassi et al., 2003) and fungi (Jankiewicz and Michniewicz, 1987; Hashimoto and Nakata, 2003; Sakamoto and Kawasaki, 2003). Yields of α-L-AFase were relatively better (88.7 nkat/mg protein) when *R. marinus* was grown in shake flasks as compared to that obtained when grown in a bioreactor (54.5 nkat/mg protein) (Gomes et al., 2000). SSF has been used successfully for α-L-AFases production from different fungi (Roche et al., 1995; Filho et al., 1996; Huerta-Ochoa et al., 2003). SSF system resembles the natural habitat of microbes and, therefore, may prove efficient in producing certain enzymes and metabolites. However, not much is known about α-L-AFases production by bacteria using SSF.

**1.4.5 Biochemical properties of α-L-AFases**

Microbial α-L-AFases vary in their molecular masses, which can be as high as 495 kDa for α-L-AFase from *Streptomyces purpurascens* IFO3389 (Komae et al., 1982) (Table 1.1). Multiple forms of α-L-AFase have been detected in the culture broth of some fungi such as *Aspergillus awamori* (Kaneko et al., 1998a), *A. nidulans* (Ramon et al., 1993), *A. niger* (Rombouts et al., 1988), *A. terreus* (Luonteri et al., 1995), *Penicillium capsulatum* (Filho et al., 1996), *P. purpurogenun* and *Sclerotina fructigena* (Laborda et al., 1973). In contrast, few bacteria produce multiple forms of α-L-AFase including *Streptomyces diastaticus* (Tajana et al., 1992).
Table 1.1: Microbial α-L-arabinofuranosidases: some biochemical characteristics and substrate specificity

<table>
<thead>
<tr>
<th>Micobial source</th>
<th>Mw kDa</th>
<th>Opt. pH</th>
<th>Opt. Temp.°C</th>
<th>$K_m$ mM</th>
<th>$V_{max}$ U/mg</th>
<th>Active on Substrates</th>
<th>References</th>
</tr>
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<tbody>
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</tr>
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<td>65</td>
<td>6.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>Weinstein and Albersheim, (1979)</td>
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<td>110</td>
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<td>60</td>
<td>ND</td>
<td>ND</td>
<td>AOS, BA, not active on AX, AG</td>
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<td>AOS, not active DA, AX, AG</td>
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<td>749</td>
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<td>5.6 - 6.2</td>
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<td>555</td>
<td>AX,OSX</td>
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<td>52.9</td>
<td>ND</td>
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<td>1.19</td>
<td>26.1</td>
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References:
- De Ioannes *et al.*, (2000); Carvallo *et al.*, (2003)
- Filho *et al.*, (1996)
- Fritz *et al.*, (2008)
- Nogawa *et al.*, (1999)
- Poutanen, (1988a)
- Poutanen, (1988b); Margolles-Clark *et al.*, (1996)
- Baker *et al.*, (1979)
- Fielding and Byrde, (1969)
- Akinrefon, (1968)
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The effect of temperature and pH on the α-L-AFase depends on the source from which the enzyme is isolated (Table 1.1). The highest temperature stability has been obtained for α-L-AFase from *Thermotoga maritima* MSB8. This cloned enzyme has an optimal temperature of 90°C at pH 7. At 90°C and pH 7, the enzyme was stable for 24 h. It also retains 50% of its activity at 100°C over a period of 20 min (Miyazaki, 2005). The other example is α-L-AFase from *R. marinus*, which is stable at 85°C for 8.3 h in a pH range of 5.0–9.0 (Gomes et al., 2000; Margolles and Reyes-Gavilán, 2003). As shown in Table 1.1, generally the microbial α-L-AFases have a broad range of pH and temperature dependence, with optimal activities occurring between pH 3.0–6.9 and 40–75°C (Kaji, 1984; Lee and Forsberg, 1987; Bezalel et al., 1993; Fernández-Espinar et al., 1994; Filho et al., 1996; Saha and Bothast, 1998b). However, reported fungal α-L-AFase tend to be more acidophilic than bacterial α-L-AFases (Table 1.1). The purified enzyme from *Rhodotorula flava* is highly acid stable, retaining 82% of its activity after being maintained for 24 h at pH 1.5 and at 30°C (Uesaka et al., 1978). Optimum activity of this enzyme is at pH 2.0. The α-L-AFase from *Corticium rolfsii* had an optimum activity at pH 2.5 toward beet arabinan (Kaji and Yoshihara, 1971). The AF from *Talaromyces emersonii* is showed a pH and temperature optima of 3.2 and 70°C, respectively (Tuohy et al., 1994).

The activities of α-L-AFases are affected by metal ions, ionic and nonionic detergents, and chelating and reducing agents depending on the enzyme and concentration of the agent used (Hespell and O'Bryan, 1992; Kormelink and Voragen, 1993). For instance, the activities of α-L-AFase (abfB) from *Bifidobacterium longum* B667 (Margolles and Reyes-Gavilán, 2003) and
α-L-AFase (AbfD3) from *Thermobacillus xylanilyticus* D3 (Debeche *et al.*, 2000) were not affected by EDTA, DTT, but were affected by Cu$^{2+}$ ions. Metal ions such as Ag$^{2+}$, Hg$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Co$^{2+}$ and Ni had an inhibitory effect on some of these enzymes (Tsujibo *et al.*, 2002; Margolles and Reyes-Gavilán, 2003; Sakamoto and Kawasaki, 2003).

### 1.4.6 Classification of arabinose-releasing enzymes

The enzymes hydrolyzing L-arabinose linkages have been classified based on different criteria.

#### 1.4.6.1 Enzyme commission classification

According to the enzyme commission, arabinan-degrading enzymes were classified on the basis of their mode of action into exo-acting α-L-arabinofuranosidases or α-arabinosidases (EC 3.2.1.55) and endo acting 1→5-α-L-arabinan 1→5-α-L-arabinanohydrolases (EC 3.2.1.99), more commonly called endo 1→5-α-L-arabinanases.

#### 1.4.6.2 Kaji (1984) classification

Kaji (1984) subdivided α-L-arabinofuranosidases into two groups on the basis of the microbial sources and substrate specificity of the enzyme. These two groups included type A and type B enzymes which have been isolated from *Streptomyces purpurascens* and *Aspergillus niger*, respectively (Komae *et al.*, 1982; Rombouts *et al.*, 1988; Voragen *et al.*, 1988; Pitson *et al.*, 1996). Type A α-L-arabinofuranosidases act only on low molecular weight substrates, such as p-nitrophenyl-α-L-arabinofuranoside (pNPAF) and L-arabino-oligosaccharides, and are inactive against polymers. Type B α-L-arabinofuranosidases are active on the side-chain L-arabinosyl residues of L-arabinan, L-arabinoxylan, and
L-arabinogalactan, but also hydrolyze simple synthetic substrates. Type A-α-L-AFses belong to GH 51 (Weinstein and Albersheim, 1979; Komae et al., 1982; Sakamoto and Thibault, 2001; Sakamoto and Kwasaki, 2003), whereas, Type B-α-L-AFses belong to glycoside hydrolase 54 (Sakamoto and Thibault, 2001; Miyanaga et al., 2004). Kaji (1984) classification became ineffective because subsequently isolated enzymes showed different properties which could not be assigned to any one of the two types mentioned above. These enzymes, called arabinoxylan arabinofuranohydrolases ((1→4)-[β-D-arabinoxylan] arabinofuranohydrolase), are specifically active on arabinofuranosidic linkages in arabinoxylans from oat spelt, wheat or barley, and have no activity toward pNPA (Kormelink et al., 1991; Van laere et al., 1997). These enzymes specifically cleave arabinofuranose from single-substituted xylopyranosyl residues in the xylan backbone, and have only low activity against substrates like pNPAF or branched arabinan. AXH from Aspergillus awamori is an example of this type of arabinofuranosidase (Kormelink et al., 1991; Kormelink et al., 1993). Similar enzymes from Bifidobacterium adolescentis (Van Laere et al., 1997) and from Trichoderma reesei have also been isolated that have a slightly different specificity since they are active against arabinofuranosyl groups linked to double-substituted xylopyranosyl residues, and are therefore termed AXH-d (Pitson et al., 1996).

1.4.6.3 Beldman et al. (1997) classification:

Beldman et al. (1997) classified arabinose-releasing enzymes into six groups based on the substrate specificity and mode of action of these enzymes. These are (1) α-L-arabinofuranosidases (E.C 3.2.1.55) that are not active toward polymers, and show activity against α-1,5-L-arabinofuranooligosaccharides
oligomers from arabinan, arabinoxylan, arabinogalactan as well as pNPAF (Weinstein and Albersheim, 1979; Komae et al., 1982), (2) α-L-arabinofuranosidas which are active toward polymers as well as oligomers from arabinan, arabinoxylan and arabinogalactan and pNPAF (Kaji and Tagawa, 1970; Rombouts et al., 1988), (3) α-L-arabinofuranohydrolases specific for arabinoxylan from oat spelts, barley and wheat but show no activity against pNPAF (Kormelink et al., 1991; Van Laere et al., 1997), (4) Exo-α-L-arabinanases which are not active toward pNPAF but act in exo manner on the side chains of branched arabinan, (5) β-L-arabinopyranosidases which are active only against p-nitrophenyl-β-L-arabinopyranoside, and (6) Endo-1→5-α-L-arabinanase (E.C.3.2.1.99) which are active in degrading linear 1→5-α-L-arabinan.

1.4.6.4 Numan and Bhosle (2006) classification

Kaji (1984) classified α-L-AFases based on their sources and substrate specificity while Beldman et al. (1997) classified arabinose-releasing enzymes depending on the mode of action and their substrate specificity. However, both classifications were ineffective as they were too broad to define the substrate specificities of these enzymes. Moreover, newly isolated enzymes have shown different modes of actions than those previously used for enzymes classification (Kaji, 1984; Beldman et al., 1997). Because of this, further subclasses and a new class needs to be added to the existing system of classification proposed by Beldman et al. (1997). In view of this, Numan and Bhosle (2006) introduced three new subclasses for the existing arabinoxylan-α-L-arabinofuranohydrolases and designated Subclass (1) AXHB-md 2, 3, Subclass (2) AXHB-m 2,3 and Subclass (3) AXHd3 (Table 1.2). Subclass (1) AXHB-md
Subclass (1) AXHB-md 2,3 includes enzymes that release arabinose from both singly and doubly substituted xylose, and are able to hydrolyze p-nitrophenyl α-L-arabinofuranoside at a rate similar to that for oligosaccharide substrates. This subclass was exemplified by the enzyme arabinoxylan arabinofuranohydrolase isolated from germinated barley (Ferré et al., 2000). Subclass (2) AXHB-m 2,3 includes enzymes that hydrolyze arabinose residues from C2 or C3 linked to a single-substituted xylose residue and do not hydrolyze p-nitrophenyl α-L-arabinofuranoside. The enzyme isolated from Bifidobacterium adolescentis (Van laere et al., 1997) represents this subclass. Subclass (3) AXHd3 includes enzymes that are able to release only C3-linked arabinose residues from double-substituted xylose residues but do not hydrolyze p-nitrophenyl-α-L-arabinofuranoside. This subclass was represented by the enzyme isolated from B. adolescentis (Van Laere et al., 1999).

Recently, new types of α-L-AFases have been isolated with properties that have not been reported earlier. Such enzymes could not be assigned to any of the arabinose-releasing enzyme classes. These enzymes have the ability to act on both interior α-1,5 backbone and α-1,3—side chains of arabinan and debranched arabinans. In addition, they are able to act on p-nitrophenyl α-L-arabinofuranoside and some were also able to act on p-nitrophenyl β-L-arabinopyranoside. In view of this, Numan and Bhosle (2006) assigned these enzymes into two new subclasses namely TB AFase and Tm AFase which are belonging to Type C α-L-arabinofuranosidase. The enzymes isolated from the thermophilic bacterium PRI-1686 (Birgisson et al., 2004) and from the hyperthermophilic bacterium Thermotoga maritima MSB8 representes these subclasses (Miyazaki, 2005) (Table 1.2).
Table 1.2: Classification of arabinose-releasing enzymes based on Numan and Bhosle (2006).

<table>
<thead>
<tr>
<th>Enzyme Class</th>
<th>Enzyme subclass</th>
<th>pNPAP</th>
<th>pNPAF</th>
<th>Arabinan</th>
<th>Arabinogalactan</th>
<th>Arabinoxylan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type A α-L-Arabinoferanosidase</td>
<td>α-L-Arabinoferanosidase</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Type B α-L-Arabinoferanosidase</td>
<td>α-L-Arabinoferanosidase</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Type C α-L-Arabinoferanosidase</td>
<td>Tm-AFase</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>TB-AFase</td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-L-Arabinoferanohydolase</td>
<td>AXH m,d (2,3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AXHm (2,3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AXHd (C3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>β-L-Arabinopyranosidase</td>
<td>β-L-Arabinopyranosidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Exo-α-L-arabinanase</td>
<td>Exo-α-L-arabinanase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Endo-α-L-arabinanase</td>
<td>Endo-α-L-arabinanase</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

pNPAP: p-nitrophenyl-β-L-arabinopyranoside;  pNPAF: p-nitrophenyl-α-L-arabinofuranoside;
O: Oligomeric;  P: polymeric
1.4.6.5 Classification at molecular level

The most recent classification scheme based on amino acid sequences, primary structure similarities and hydrophobic cluster analysis has classified α-L-AFases into five glycosyl hydrolases families (GHs), i.e., GH3, GH43, GH51, GH54, and GH62 (Coutinho and Henrissat, 1999; Henrissat and Davies, 2000). This classification is useful to study evolutionary relationship, mechanistic information and structural features of these enzymes (Davies and Henrissat, 1995).

1.4.7 Mechanisms of action of α-L-AFases

Like other glycoside hydrolases, 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 (Zechel and Withers, 2000; de Groot et al., 2003). In both mechanisms, as shown in Figure 1.5, the hydrolysis usually requires two carboxylic acids, which are conserved within each glycoside hydrolases family (Rye and Withers, 2000) and proceed through an exocarbonium ion-like transition state (Piston et al., 1996; Rye and Withers, 2000; Shallom et al., 2002). Retaining α-L-AFases are members of GH3, GH51 and GH54 families that cleave the glycosidic bond using a two-step double-displacement mechanism, as shown in Figure 1.5a.

This was also confirmed by the crystal structure studies and snapshots along the reaction pathway of GH51 described by Høvel et al. (2003). 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.

Fig. 1.5: General mechanisms for (a) retaining and (b) inverting glycosidases. Adapted from Rye and Withers (2000).
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 (Ferchichi et al., 2003; Hövel et al., 2003). Inverting α-L-AFases representing GH43 family uses a single displacement mechanism. In this mechanism one carboxylate acts as a general base catalyst, deprotonating the nucleophilic water molecule that attacks the bond, while the other carboxylic acid acts as general acid catalyst by protonating the leaving a glycone (Fig. 1.5b) (Zechel and Withers, 2000; Shallom et al., 2002).

1.4.8 The Glycoside hydrolases families of α-L-AFases

To date, there are more than 110 amino acid sequences of different α-L-AFase. Based on amino acid sequence homology, α-L-AFase are classified into five glycoside hydrolase families (GHs). These included GH3, GH43, GH51, GH54 and GH63 (Bourne and Henrissat, 2001; Shallom et al., 2002b; CAZY classification; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Although much work has been done on the substrate specifies of these enzymes, there are only a few studies dealing with the biochemical mechanism and catalytic properties of these hydrolase families (Debeche et al., 2002; Shallom et al., 2002a; b).

Family GH51

The majority of the known α-L-AFases are belonging to family 51 of the glycosyl hydrolase classification system. These enzymes only hydrolyze small substrates, including short-chain arabino-oligosaccharides. This family is exclusively composed of retaining arabinofuranosidases which catalyse
hydrolysis via a double displacement mechanism (Ferchichi et al., 2003). This family contains genes from both taxonomic ranges and all enzymes catalyze only the hydrolysis of L-arabinofuranose residues. Currently there are 32 sequences of α-L-AFases classified as GH51 glycoside hydrolases, (Coulinho and Henrisst, 2000; Shallom et al., 2002b). Studies on family 51 α-L-AFases, from *Thermobacillus xylanilyticus* (AbfD3) have led to the localization of its two catalytic residues. These are Glu\(^{176}\) that represents the acid-base catalyst and Glu\(^{298}\) that represents the nucleophile (Debeche et al., 2000; Debeche et al., 2002). In contrast, the catalytic residues of family 51 α-L-AFase from *Geobacillus stearothermophilus* T-6 (AbfA) were recently identified as Glu\(^{175}\) (the acid-base catalyst) and Glu\(^{294}\) (the nucleophile) (Shallom et al., 2002a; b; Hövel et al., 2003). As family 51 α-L-AFases show different substrate recognition features, Juers et al. (1999) have partly attributed this rich functional diversity to the presence of additional, non-catalytic domains which, when combined with the common (β/α)\(_8\) scaffold, modify the overall active structure. Another study on (AbfD3) revealed a third glutamate (Glu\(^{28}\)) which is not directly involved in catalysis. Nevertheless, Glu\(^{28}\) is critical for normal hydrolytic activity as it might be involved in the stabilization of a catalytic transition state. The Glu\(^{28}\) is contained within an arabinofuranosidase-associated motif, and functionally homologous to a conserved residue found in exo-enzymes from both family 1 and, especially, family 5. The study suggested that Glu\(^{28}\) contributes to the overall pocket structure which determines exo-activity in family 51 arabinofuranosidases (Ferchichi et al., 2003). *Streptomyces chartreusis* GS901 AFases belonging to GH 51 consists of a central catalytic domain Glu\(^{380}\) and Glu\(^{467}\). Additionally they have unknown functional domains
existing in the N-terminal and C-terminal regions (Matsuo et al., 2000). Glu\textsuperscript{194} and Glu\textsuperscript{321} comprise the key catalytic acid-base and nucleophile residues, respectively of Abf51A from *Pseudomonas cellulosa* (Beylot et al., 2001 a; b).

**Family GH54**

The enzymes of this family are able to hydrolyze polymeric substrates such as arabininoxylans in addition to small substrates (Beldman et al., 1993; Miyanaga et al., 2004). This enzymes were shown to cleave the glycosidic bond by the same mechanism as family GH 51 with retention of the anomeric configuration, but it contain genes only from eukaryotes and all enzymes of this family catalyze only the hydrolysis of L-arabinofuranose residues.

**Family GH3** are retaining enzymes, whereas **family GH43** are inverting ones (Coutinho and Henrissate, 2000). Family GH43 which work via the inverting mechanism, and they are mainly bifunctional α-L-arabinofuranosidases/β-xylosidases (EC 3.2.1.37) able to hydrolze terminal non-reducing D-xylose, but also α-L-arabinofuranose residues from α-L-arabinosides. Member of this family originate from both eukaryotic and prokaryotic sources. The stereochemistry of **family GH62** is not characterized (Shallome et al., 2002a; Miyanaga et al., 2004).

**1.4.9 Substrate specificity of α-L-AFases**

Microorganisms produce α-L-arabinofuranosidases, which show different specificities in hydrolysis of arabinose containing polysaccharides and oligosaccharides as well as synthetic substrates (Table. 1.1). Some of these enzymes also act on some arabinose containing substrates in unexpected manner. For instance, the α-L-AFase Abf D3 from *Thermobacillus xylaniliticus*
was specifically active towards the furanosidic conformation, and \(\alpha\)-linkages. However, as a member of GH51, Abf D3 would be expected to have a low activity towards arabinoxylans, but was found to be extremely active on wheat arabinoxylan, larch xylan, and oat spelt xylan (Debeche et al., 2000). Furthermore, the two \(\alpha\)-L-AFases (AFQ1 and AFS) from *Penicillium chrysogenum* preferred 1,2- or 1,3-linked arabinofuranose residues attached as side chains to the linear \(\alpha\)-1,5-L-arabinan. Both of enzymes also showed much larger activity on sugar beet L-arabinan than on debranched arabinan and could not hydrolyze Larch wood arabinogalactan. However, AFQ1 easily degraded arabinoxylan, AFS1 whereas could do so only after prolonged incubation period of 6 h. Furthermore, AFS1 hydrolyzed soybean arabinogalactan more easily than did AFQ1 (Sakamoto and Kwasaki, 2003). The best substrate for \(\alpha\)-L-AFase form *Bifidobacterium breve* K-110 was pNPAF, followed by ginsenoside Rc which was hydrolyzed into ginsenoside Rd. Unexpectedly, the enzyme also did hydrolyse \(p\)-nitrophenyl-\(\beta\)-galactopyranoside, \(p\)-nitrophenyl-\(\beta\)-xylopyranoside, \(p\)-nitrophenyl-\(\beta\)-fucopyranoside, (Shin et al., 2003).

Kormelink et al. (1991) described another type of \(\alpha\)-L-AFase (AXH) from *A. awamori* that was highly specific for arabinoxylans, and unlike other \(\alpha\)-L-AFases, did not show any activity towards \(p\)NP-\(\alpha\)-L-arabinofuranoside, arabinans, and arabinogalactans. Arabinoxylan-derived oligosaccharides were treated with AXH from *A. awamori* and two types of \(\alpha\)-L-AFase from *A. niger* (Kormelink et al., 1993). All these enzymes acted on arabinoxylan oligosaccharides. Van Laere et al. (1997) described a new arabinofuranohydrolase from *B. adolescentis* able to remove arabinosyl residues from double-substituted xylose units in arabinoxylan. The enzyme showed no
activity toward sugar beet arabinan, soy arabinogalactan, arabinooligosaccharides, and arabinogalactooligosaccharides. *Streptomyces thermoviolaces* expresses (STX-IV) a gene coded for α-L-AFase which exhibited similar substrates specificity to that of enzymes from *Streptomyces lividance*, *A. awamori* and *Clostridium stercorarium*. These enzymes preferred arabinoxylan but not other arabinosaccharides and showed a low activity with pNPAF (Tsujibo et al., 2002). The α-L-AFase I from *A. awamori* preferentially hydrolyzed the (1→5) linkage of branched arabinotrisaccharide, whereas α-L-AFase II from the same organism preferentially hydrolyzed (1→3) linkage in the same substrate (Kaneko et al., 1998a). The α-L-AFase I released arabinose from the nonreducing terminus of arabinan, whereas α-L-AFase II preferentially hydrolyzed the arabinosyl side chain linkage of arabinan. A novel property of α-L-AFase from *A. awamori* was its capacity to release a substantial portion (42%) of feruloyl arabinose from intact wheat straw arabinoxylan (Wood and McCrae, 1996).

### 1.4.10 Molecular biology of α-L-AFases

Some α-L-AFases have been studied up to molecular level. The genes coding for these enzymes have been identified, cloned and expressed in different bacterial and fungal systems. In addition, the protein products of these genes have been sequenced and the evolutionary relationship among some of the sequenced proteins has been reported using the phylogenetic tree analysis (Degrassi et al., 2003). For example, some of the cloned genes, i.e., STX-IV from *Streptomyces thermoviolaces* OPC-520 chromosome (Tsujibo et al., 2002), AkabfA and AkabfB from *A. kawachii* and AwabfA and AwabfB from
A. awamori (Koseki et al., 2003; 2006), xarB from the thermophilic anaerobe *Thermoanaerobacter ethanolicus* JW200 (Mai et al., 2000), α-L-AFase gene from *B. longum* B667 (Margolles and Reyes-Gavilán, 2003), *Bifidobacterium breve* K-110 (Shin et al., 2003), and from the *Clostridium cellulovorans* genomic library (Kosugi et al., 2002) have been characterized. Similarly, genes such as Abf51A from the genomic library of *P. cellulosa* (Beylot et al., 2001a; b), abf1 from *P. purpurogenum* (Carvallo et al., 2003), abfA from *T. maritima* TM0281 (Miyazaki, 2005) and abfB from *Fusarium oxysporum f. sp. dianthi* (Fod) (Chacón-Marténez et al., 2004) have been well characterized. Other α-L-AFases coding genes that have been isolated, sequenced and/or expressed in different hosts included ABF2 of *A. niger* (Crous et al., 1996a), AFase of *A. nidulans* (Sanchez-Torres et al., 1996; 1998), AFase of *T. reesei* RutC-30 (Margolles-Clark et al., 1996), arfB of *Clostridium stercorarium* (Schwarz et al., 1995); arfI and arfII of *C. xylanolytica* (Kim et al., 1998); AF of *Aspergillus sojae* (Kimura et al., 2000); abfD3 from *T. xylanilyticus* (Debeche et al., 2000) and AFase of *P. ruminicola* B14 (Gasparic et al., 1995a; b). Furthermore, Sakka et al. (1993) reported the nucleotide sequence of the *C. stercorarium* xylA gene encoding a bifunctional protein with β-D-xylosidase and α-L-AFase activities. The genes encoding the enzyme arabinoxylan arabinofuranohydrolase, which releases arabinose from arabinoxylan, have been cloned from the closely related fungi *A. niger* and *Aspergillus tubingensis* (Gielkens et al., 1997).

1.4.11 Substrate binding domain

Amino acid sequencing as well as crystal structure studies indicate the presence of substrate-binding domain (SBD) in some of the reported enzymes. The SBD
may take part in the efficiency of the enzyme function (Kuno et al., 1998). However, the possible roles of SBD of α-L-AFases in the release of arabinofuranosyl residues is not yet clear (Kimura et al., 2000). Some α-L-AFases with SBD have been reported, i.e., α-L-AFase from *Streptomyces lividans* has a cellulose-binding domain (CBD) (Vincent et al., 1997). Other α-L-AFases such as those produced by *Streptomyces chartreusis* GS901 possess additional functional domains at both the N-terminal and the C-terminal regions. However, these domains did not show any similarities to the known SBD observed in many other types of glycanases. It might represent a novel kind of SBD (Matuso et al., 2000). *Aspergillus kawachii* IFO4308 α-L-AFase (AkAbfB) was found to have an arabinose-binding domain (ABD) that showed a number of distinct characteristics that are different from those of carbohydrate-binding module (CBM) (Miyanaga et al., 2004). Recently, Bolam et al. (2004) showed that the X4 modules from a *Cellvibrio japonicus* α-L-AFase (Abf62A) binds to polysaccharides. This protein comprises a new family of CBMs, designated as Abf62A-CBM35. There are more than 13 α-L-AFases that have been grouped in family 42 of CBM (Coutinho and Henrissat, 1999).

1.4.12 The crystal and three dimensional structure of α-L-arabinofuranosidase

So far, only three α-L-AFases have been studied for their three-dimensional structure. There appears considerable diversity in the three-dimensional structure of these enzymes. These enzymes were α-L-AFase B (AkabfB) (EC 3.2.1.55) from *A. kawachii* IFO 4308 located within GH 54 family (Miyanaga et al., 2004), α-L-AFase (AbfA) (EC 3.2.1.55) from *G. stearothermophilus* T-6 located within GH 51 family (Shallom et al., 2002;
Hövel et al., 2003) and the bifunctional xylanase D/ α-L-arabinofuranosidase (XynD)/(Xyn43A) (EC 3.2.1.8 and EC 3.2.1.55, respectively) of Paenibacillus polymyxa located within GH 43 family (Hövel et al., 2003).

1.5 Biotechnological applications of α-L-AFases

The importance of lignocellulose-degrading enzymes is well defined because of their role in many industrial and biotechnological processes. This resulted in re-establishment of a new era for the efficient utilization of the cheap agricultural waste materials. α-L-AFases, with their synergistic action with other lignocellulose-degrading enzymes, are the promising tools in various agro-industrial processes (Aryon et al., 1987; Saha, 2000). These include production of important medicinal compounds, improvement of the wine flavors, bread quality, pulp treatment, juice clarification, quality of animal feedstock, production of bioethanol and the synthesis of oligosaccharides.

1.5.1 Production of arabinose as antiglycemic agent

Recently, there is a growing interest for L-arabinose as a possible food additive because of its sweet taste, and its low uptake due to its poor absorption by the human body (Matuso et al., 2000). Moreover, it has been proved that L-arabinose selectively inhibits intestinal sucrase in a competitive manner and thus reduces the glycemic response after sucrose ingestion in animals (Seri et al., 1996). Studies carried out on mice suggest that L-arabinose dose-dependently suppressed the increase of blood glucose level after the ingestion of sucrose (Shin et al., 2003). Furthermore, L-arabinose delays and reduces the digestion, absorption and the net energy derived from sucrose when both are ingested simultaneously. Based on these findings, L-arabinose can be
used as a physiologically functional sugar that inhibits sucrose digestion. In this way, L-arabinose is useful in preventing postprandial hyperglycemia in diabetic patients (Sanai et al., 1997). Therefore, effective L-arabinose production is a vital perquisite for its use in this respect as well as for its importance in food industry. To achieve this goal, it is necessary to use arabinose-releasing enzymes α-L-AFases, and defined polysaccharides and oligosaccharides from different agricultural raw materials (Matuso et al., 2000; Takao et al., 2002; Rahman et al., 2003).

1.5.2 Production of antimetastatic and anticarcinogenic compounds

Ginsenosides Rb2 and Rc are the main components of ginseng (the root of Panax ginseng C.A. Meyer, Araliaceae). These roots are frequently used as a traditional medicine in China, Korea, Japan and other Asian countries. Ginsenosides Rb2 and Rc are L-arabinofuranoside- and L-arabinopyranoside-bound glycosides, respectively, in ginsenoside Rd (Shin et al., 2003). These ginsenosides are transformed to compound K, via ginsenoside Rd, by intestinal bacteria in human intestine by the action of α-L-AFase (Bae et al., 2000) (Fig. 1.6).

![Proposed metabolic conversions for the ginsenoside Rb2 by α-L-arabinofuranosidase from B. breve K-110. Modified from Shin et al. (2003).](image)

**Fig. 1.6**: Proposed metabolic conversions for the ginsenoside Rb2 by α-L-arabinofuranosidase from *B. breve* K-110. Modified from Shin et al. (2003).
The pharmacological actions of these ginsenosides have been explained based on the biotransformation of ginsenosides by glycosidases of human intestinal bacteria (Hasegawa et al., 1997; Wakabayashi et al., 1997; Akao et al., 1998; Bae et al., 2000; 2002). The latter bacteria utilize α-L-AFase to transform the protopanaxadiol ginsenosides to compound K that exhibits antimetastatic and/or anticarcinogenic effects. Moreover, compound K can be produced effectively by different arabinosidases including α-L-AFases and α-L-arabinopyranosidase (Kaji and Tagawa, 1970).

1.5.3 α-L-AFases and wine industry

One of the most important characteristics of wine quality is its aromatic fragrance. It is now well established that certain monoterpenes contribute significantly to the flavor of wine (Mateo and Jiménez, 2000). Terpenols are strongly aromatic molecules that represent an important part of aromas (Giunata et al., 1988). They are not volatile and are directly accessible to the olfactory mucosa (Vorin et al., 1990; Winterhalter, 1990; Biskup et al., 1993). A major portion of these monoterpenols in grapes musts, wines, other alcoholic beverages (brandy, bitters, etc.) and fruit juices (apple, apricot, peach, papaya, passion fruit etc.) (Schwab et al., 1990; Biskup et al., 1993) are linked to disaccharide moieties, in which the major terminal non-reducing sugar is α-L-arabinofuranose which can be released by the action of α-L-AFases (Biskup et al., 1993). It is now clear that the glycosidically bound volatiles can be released by sequential enzymatic hydrolysis in two stages. In the first step, and depending on the precursor, the glycosidic linkage is cleaved by α-L-AFases, followed by the action of the other glycosidase, which then...
liberates the monoterpenols (Fig. 1.7) (Giinata et al., 1988; 1990; Wu et al., 1990; Marlatt et al., 1992; Spagna et al., 1998; 2002).

![Figure 1.7: Mechanism of action of the glycosidase α-L-arabinofuranosidase and β-D-glucopyranosidase (βG) on diglycosidic precursors. ROH is a volatile aglycone such as monoterpenols and other alcohols. Modified from Spagna et al. (1998).](image)

Thus, α-L-AFases treatment followed by the addition of other glycosidases can be used for the enhancement of wine flavor by the release of free terpenols. Moreover, Yannai and Sato (2000) have reported that α-L-AFase from Pichia capsulata X91 is active at ethanol concentrations found in wine and able to release considerable amount of monoterpenols, especially linalool, citronellol and geraniol, thereby increasing the aromatic flavors of different wines. Furthermore, the immobilized α-L-AFase, β-D-glucopyranosidase and α-L-rhamnopyranosidase from A. niger increased the aroma of a model wine solution to more than 600 mg/L of total free terpenols (Spagna et al., 1998; 2002). Today, a lot of interest has been generated in the involvement of α-L-AFases in enhancing the aroma. This is mainly achieved by using the recombinant yeast strain (YCA1) [Saccharomyces cerevisiae strain T73 (CECT1894)] transformed with YCAbfB from Aspergillus niger N400 (CPS 120.49)] that was capable of efficiently secreting α-L-AFase directly in vinification process or by directly adding the purified enzyme obtained from it.
(Sanchez-Torres et al., 1996). Preliminary experiments carried out with this recombinant yeast strain (YCA1) have shown increased levels of some volatile compounds involved in wine aroma (Sanchez-Torres et al., 1996). Furthermore, during wine aging, a number of the fragrant precursors (such as linalol, nerol and geraniol) turn into less-fragrant compounds (α-terpineol, diols, and triols, oxides, etc.) so that after 6–7 months of aging for an aromatic wine (Muscato wines), the final result is often a reduction in the more fragrant-free terpenes. The addition of glycosidases to the wine increases its aroma without this disadvantage (Biskup et al., 1993). For instance, α-L-arabinofuranosidase and β-D-glucopyranosidase (βG, EC 3.2.1.21) are currently produced on an industrial scale from A. niger (Aryon et al., 1987), and are used in the aromatization of musts, wines and other alcoholic beverages (Schwab et al., 1990; Biskup et al., 1993).

1.5.4 α-L-AFases, acetic acid production and quality of the bread

Staling is probably the main problem that occurs during bread storage. This results in a decreased bread shelf life and causes serious economic losses to the bread industry (Gobbetti et al., 2000). Pentosans are important functional ingredients in bread and their positive role in bread texture and staling is well known (Casier et al., 1973; Kim and D’Appolonia, 1977; Jankiewicz and Michniewicz, 1987). Pentosans added to the dough may be moderately hydrolysed by wheat flour enzymes and especially by exogenous enzymes such as xylan degrading system including α-L-AFases (Fessas and Schiraldi, 1998; Jiménez and Martinez-Anaya, 1999). These enzymes produce free pentoses (mainly arabinose and xylose) thereby increasing the availability of soluble carbohydrates in the dough (Gobbetti et al., 1999; Jiménez and Martinez-
Anaya, 1999; Martinez-Anaya and Devesa, 1999; Gobbetti et al., 2000). This positively interferes with the metabolism of sourdough lactic acid bacterium *Lactobacillus hilgardii* (Gobbetti et al., 1999). This bacterium increases the acidification rates and the production of acetic acid. For example, Gobbetti et al. (2000) showed that by using pentosans, α-L-AFase from *A. niger* and *Lactobacillus plantarum* 20B, soluble carbohydrate availability, acidification rate and production of acetic acid increased during sourdough fermentation. α-L-AFase mainly hydrolyse the exterior arabinofuranosyl linkages of pentosan in the dough thereby making pentoses available for fermentation by *L. plantarum* (Gobbetti et al., 2000). Recently, α-L-AFases along with pentosanse and other enzymes have been considered as natural improvers that greatly enhance the overall quality of bread (Jiménez and Martinez-Anaya, 1999; Martinez-Anaya and Devesa, 1999). The enzyme treatment delayed the bread staling and increased the shelf life of the bread thereby giving economic benefits to the bread industry (Jiménez and Martinez-Anaya, 1999; Martinez-Anaya and Devesa, 1999; Gobbetti et al., 2000).

### 1.5.5 α-L-AFases in pulp and paper industry

Several commercial xylanase preparations are available for the treatment of pulp (Viikari et al., 1994). Application of α-L-AFase would further enhance the delignification of pulp as the enzyme acts to release the arabinose side chain that retards the action of other bleaching enzymes (Bezalel et al., 1993; Gübitz et al., 1997). The removal of lignin from semi-bleached kraft pulp was improved when the pulp was treated with α-L-AFase from *B. stearothermophilus* L1 together with xylanase (Bezalel et al., 1993). The enzyme acted synergistically with a thermophilic xylanase in the delignification
process, releasing 19.2% of lignin. Delignification obtained using the combined enzyme treatment exceeded the sum of the amounts obtained using the enzymes individually (Bezalel et al., 1993). According to Margolles-Clark et al. (1996), *Trichoderma reesei* RutC-30 α-L-AFase could also liberate >60% of the arabinose from arabinoglucuronoxylan isolated from pine kraft pulp. The treatment of softwood kraft pulp with the crude α-L-AFase-rich xylanase and mannanase from *R. marinus* increased the bleachability of the pulp when used in a X—Q—D—Q—P bleaching sequence, where X was enzyme treatment, Q was chelation, D was chlorine dioxide treatment with NaClO₂ in acidic solution and P was the peroxide bleaching (Gomes et al., 2000). The highest increase in brightness (1.8% ISO) was achieved when the mixture of α-L-AFase-rich xylanase and mannanase was used for the pulp treatment. The observed increase in the brightness (1.9–2.1%) was similar to the value obtained using commercial enzyme preparation (Gomes et al., 2000). The high thermal and pH stability, broad pH optima and lack of cellulose activity of the α-L-AFase, xylanases (Manelius et al., 1994; Gübitz et al., 1997) and mannanase produced by *R. marinus* are most useful for biobleaching of pulp and paper (Dahlberg et al., 1993; Gomes et al., 2000).

1.5.6 α-L-AFases and animal feedstock

The digestion of feedstuffs by ruminal microorganisms results in the production of acids and microbial cells, which provide the host animal with its main sources of energy and protein (Dehority and Scott, 1967). Although hemicelluloses (mainly xylans) represent 30–40% of the total forage carbohydrate, their contribution to dietary energy available to the animal is often decreased because of low overall (40–60%) digestion (Dehority, 1968;
Coen and Dehority, 1970; Weaver et al., 1992). The increase in digestibility of feedstuffs is well correlated with the decrease in the degree of substitution of the hemicellulose polymers with arabinosyl residues (Morrison, 1982; Greve et al., 1984). L-Arabinose residues prevent the total hydrolysis of xylans. Therefore, any mechanism able to remove the arabinosyl side chains from hemicellulose should increase its digestibility (Dehority, 1965; 1967; Coen and Dehority, 1970; Cotta, 1993; Hespell and Cotta, 1995). The utilization of cell wall polysaccharides by poultry and pigs was improved by the addition of cellulases, pectinases and xylanases (Chesson, 1987). Moreover, the addition of \( \alpha \)-L-AFases removes arabinose side groups that restrict the action of glycanases and could further promote the hydrolysis of solubilized cell wall polysaccharides (Greve et al., 1984; Hespell and O'Bryan, 1992; Kormelink and Voragen, 1993). It has been shown that the use of commercial enzymes preparation containing \( \alpha \)-L-AFases enhanced the activity of xylanase because the latter prefers unsubstituted regions of xylan as a substrate, thereby reducing the viscosity of the feedstuffs used (Mathlouthi et al., 2002). Cotta (1993) reported that \( \alpha \)-L-AFase isolated from Ruminococcus albus 8 removed arabinosyl residues from alfalfa cell wall (ACW), pectic and hemicellulosic polysaccharides, thereby making these substrates more susceptible to attack by other glycanases. For a given species, such as R. albus, digestion can vary from a low of 5 to a high of 88% for corn (Hespell and Cotta, 1995). \( \alpha \)-L-AFases helps endo-xylanases in the hydrolysis of arabinoxylan, thereby improving the feed digestibility (Campbell and Bedford, 1992; Roche et al., 1995). The addition of mixture of xylanases and \( \alpha \)-L-AFases as a strategy to increase digestion is currently being used in some countries (Roche et al., 1995). This
approach has been considered in the European Community (AIR contract number AIR1 CT92) (Roche et al., 1995). Genetic manipulation of anaerobic bacteria and ruminal organisms is yet another strategy to increase the production of xylan-degrading enzymes, which can be used to improve the digestion of plant materials (Patterson, 1989; Van laere et al., 1997; Kaneko et al., 1998a). This has to involve cloning of α-L-AFase genes into the manipulated ruminal bacteria to increase the efficiency of xylan-degrading enzymes. This approach has been proved good when the cloned α-L-AFase from P. ruminicola β14 was used (Gasparic et al., 1995a;b).

1.5.7 α-L-AFases in fruits juice industry

α-L-AFases are receiving attention for their applications in fruit juice clarification (Romboust et al., 1988). The preparations of pectinolytic enzymes utilized so far contain significant amounts of α-L-AFases (Pilnik, 1982; Winterhalter, 1990; Weaver et al., 1992). 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 (De Vries et al., 1982; Churms et al., 1983; Voragen et al., 1988). As industrial enzymes often do not require extensive purification, the juice industry can use α-L-AFases and arabinanase-containing plant extracts (Hood and Jilka, 1999; Skjat et al., 2001). For example, in apple and pear juice production, haze formation is a problem due to the presence of solubilized arabinans (Churms et al., 1983). The precipitates can most probably be avoided by adding sufficient amounts of α-L-AFase and endo-arabinanase (Whitaker, 1984; McCleary et al., 1988; Voragen et al., 1988). Birgisson et al. (2004) reported an α-L-AFase from the
thermophilic bacterium PRI-1686 belonging to the recently described phylum of Thermomicrobia. This enzyme has the ability to degrade the interior α-1,5 backbone and α-1,3-side chains of arabinan. Moreover, Miyazaki (2005) described a thermophilic α-L-AFase from the hyperthermophilic bacterium *T. maritime* MSB8 that had the ability to degrade arabinan and debranched arabinan. Such properties are useful to avoid haze formation in fruits juice industry.

1.5.8 Production of fermentable sugars for bioethanol industry

Enzyme-catalyzed conversion of sugarcane, sugar beet, corn or wheat to ethanol by distillers yeast *Saccharomyces cerevisiae* is the current process for the industrial production of bioethanol (Sørensen *et al.*, 2005). These substrates contain non-fermentable hemicelluloses. These hemicelluloses remain unutilized and accumulate as by-product residues (~70 %by weight of the total residue) during the process of ethanol production (Bacic and Stone, 1980; Adams *et al.*, 2004; Sørensen *et al.*, 2005). The utilization of these residual hemicelluloses is essential for the efficient conversion of these compounds to ethanol, value-added products and industrial chemicals (Saha, 2000; Zaldivar *et al.*, 2001; Saha, 2003). Nevertheless, these substrates require a suitable pretreatment before they can be used for the production of ethanol (Saha, 2003). For instance, acid hydrolysis can be used for the hydrolysis of arabinoxylans in hemicelluloses to monosaccharides. However, enzymatic hydrolysis is preferred due to reduced formation of byproducts that may inhibit the subsequent microbial fermentation (Saha, 2000). The complexity and heterogeneity of the arabinoxylans in hemicelluloses demand enzyme systems that convert these substrates into fermentable sugars (Filho *et al.*, 1996; Leathers, 2003; Saha,
Such an enzyme system needs to include de-polymerizing and the side-group cleaving enzymes to degrade hemicelluloses into pentoses monosaccharides (Saha, 2003). Moreover, such a system will also need a microorganism not only capable of utilizing pentoses, but also able to withstand high concentrations of ethanol produced during the process (Saha and Bothast, 1998a,b; Zaldivar et al., 2001; Leathers, 2003; Saha, 2003). Therefore, tailored enzymes are required to hydrolyse lignocellulosic substrates to fermentable sugars (Saha, 2003; Sørensen et al., 2005). The synergistic action of α-L-AFases with lignocellulose-degrading enzymes makes them potential agents for saccharifying various pretreated agricultural and forestry residues to monomeric sugars for the production of fuel and chemicals (Saha, 2000). Designed hemicellulosic enzymes consisting of Celluclast 1.5 L from *Trichoderma reesei* and Ultraflo L from *Humicola insolens* exhibited a strong synergistic interaction in catalyzing the release of xylose and arabinose from wheat arabinoxylans, which otherwise will be accumulated as by-products during the production of ethanol. This was mainly due to the cooperative action of α-L-AFases, endo-1,4-xylanases and xylosidase present in the two enzyme preparations (Sørensen et al., 2003;2005;2006a;b) . Moreover, Sørensen et al. (2005) suggested that such synergistic interaction might be useful for the production of efficient enzyme cocktails to improve the utilization of wheat hemicellulose byproducts produced during the production of ethanol (Sørensen et al., 2005). Furthermore, Saha and Bothast (1998a) suggested that the high activity of the α-L-AFase from *Aureobasidium pullulans* on both arabinan and debranched arabinan, its ability to release L-arabinose from arabinoxylans, and its high thermostability make this enzyme a promising candidate for the
production of fermentable sugars from hemicellulosic biomass for ethanol production (Saha and Bothast, 1998a).

1.5.9 Synthesis of pentose-containing compounds

Enzymes are being adopted for the synthesis of oligosaccharides and glycoconjugates via enzymatic or mixed chemo-enzymatic routes. The glycoside hydrolases (EC 3.2.1) and glycosyltransferases (EC 3.2.4) are promising enzymes as they play an important role in the synthesis strategies by performing glycosylation in one stereoselective step. Glycoside hydrolases (mainly exo-acting hydrolases) often display more relaxed regioselectivity, and unlike glycosyltransferases, an extensive palette of glycoside hydrolases, displaying a wide range of sugar specificities, are available (Ferchichi et al., 2003). Some α-L-AFases are robust and thermostable and do not require the use of costly sugar donors. For example, thermostable α-L-AFase (AbfD3) from T. xylanilyticus (Debeche et al., 2000) has the ability to catalyze transglycosylation in the presence of p-nitrophenyl α-L-arabinofuranoside and various alcohols. Moreover, Rémont et al. (2004) reported the synthesis of several pentose-containing oligosaccharides using this enzyme. The enzyme AbfD3 possessed the ability to synthesize oligosaccharides in kinetically controlled transglycosylation reactions. The products of these reactions could be useful analytic tools as reference compounds for the analysis of hemicellulase action, and for raising antibodies to well-defined motifs for immunochemical-based analysis of plant cell walls (Rémont et al., 2002; Rémont et al., 2004). Moreover, α-L-AFases that display transglycosylation ability constitute potentially interesting tools for chemoenzymatic synthesis of arabinose-
containing compounds that are difficult to access via organic synthetic methods (Rémond et al., 2004).

1.6 Scope and objectives of the present research

Looking at the importance and potentials of α-L-AFases in several biotechnological applications, it is perceived that research into exploring many aspects of α-L-AFases of desirable property is of a priority. Achieving efficient breakdown of the plant cell wall polysaccharide hemicelluloses and pectins represents an important and lucrative goal for biotechnologists. Thus, studies on the synergistic effects of the robust enzyme on the action of other hemicellulases and pectinases may lead to improvement of many existing industrial products. Moreover, isolation and characterization of robust α-L-AFases will likely have significant implications in the design of industrial processes that can be accomplished within a wide range of conditions and in commercial production of biomass-degrading enzymes. Economic production of such important industrial enzymes requires the exploring of efficient cultivation methods and cheap carbon sources. Previous studies on α-L-AFases considered neither the economic nor the environmental aspects in the production of these enzymes. Furthermore, studies on bacterial α-L-AFases are restricted to the enzymes that were produced under SmF using expensive polymeric substrates. The impact of catabolite repression on bacterial production of enzymes is known for some enzymes such as amylase. However, specific studies on the effect of catabolite repression on α-L-AFase production by bacteria grown under SSF and SmF are not available. Most of the reported α-L-AFases are less stable at elevated temperature and has a narrow range of pH stability.
Nevertheless, a few of the reported α-L-AFases were thermostable at high temperature. However, their substrate specificities were limited to one or two substrates. In fact, in addition to α-L-AFase activity and stability at acceptable ranges of temperature and pH, the enzyme activity on broad substrates is another criterion for good industrial enzymes. Exploring new ecological niche for bacteria that produce α-L-AFase may provide potent α-L-AFase microbial producers. Furthermore, earlier studies proved the efficiency of marine bacteria in production of xylanases. In view of this, there is a need to search for bacterial α-L-AFases from marine environment and their characterization, especially those with potent properties. In order to achieve this goal the present research was planned with the following major objectives.

**Objectives of the Research:**

I. Isolation and characterization of marine bacteria with ability to utilize arabinose-containing polymers.
II. Screening the isolated marine bacteria for the production of arabinofuranosidase enzyme.
III. Optimization of growth conditions for optimal production of arabinofuranosidase by selected bacteria.
IV. Isolation, purification and characterization of arabinofuranosidase.