

**STUDIES ON  
ARABINOFURANOSIDASE  
FROM MARINE BACTERIA**

**THESIS SUBMITTED TO THE GOA UNIVERSITY**

**FOR THE DEGREE OF**

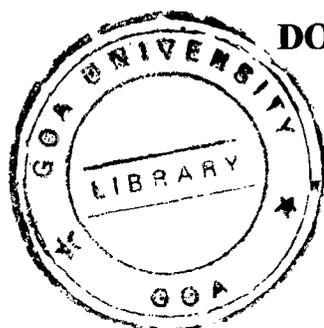
**DOCTOR OF PHILOSOPHY**

**IN**

**MICROBIOLOGY**

**BY**

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September 2008

422

..... *DEDICATED*

*TO MY BELOVED PARENTS*

# STATEMENT

As required under the University Ordinance 0.19.8 (vi), I state that the present thesis entitled "Studies on arabinofuranosidase from marine bacteria" is my original contribution and the same has not been submitted on any previous occasion. For the best of my knowledge, the present work is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been appropriately cited. Due acknowledgements has been made wherever facilities and suggestions has been availed of.

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# CERTIFICATE

This is to certify that the thesis entitled "Studies on arabinofuranosidase from marine bacteria", submitted by Mr. Mondher Thabit Abdo Numan, for the award of the degree of Doctor of Philosophy in Microbiology is based on his original studies carried out by him under my supervision. The thesis or any part therefore has not been previously submitted for any degree or diploma in any universities or institutions.

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# CONTENTS

<b>CHAPTER 1</b>	<b>Introduction and Literatures Review .....</b>	<b>1</b>
1.1	Introduction	1
1.2	Plant Cell wall polysaccharides	2
1.3	Degradation of hemicelluloses and pectins	9
1.4	The $\alpha$ -L-arabinofuranosidases ( $\alpha$ -L-AFases)	11
1.4.1	The synergistic role of $\alpha$ -L-AFases	12
1.4.2	Bifunctional $\alpha$ -L-arabinofuranosidase	14
1.4.3	Regulation of biosynthesis of microbial $\alpha$ -L-AFases	18
1.4.4	Microbial production of $\alpha$ -L-AFases	21
1.4.5	Biochemical properties of $\alpha$ -L-AFases	26
1.4.6	Classification of arabinose-releasing enzymes	35
1.4.7	Mechanisms of action of $\alpha$ -L-AFases	40
1.4.8	The Glycoside Hydrolases (GHs) families of $\alpha$ -L-AFases	42
1.4.9	Substrate specificity of $\alpha$ -L-AFases	44
1.4.10	Molecular biology of $\alpha$ -L-AFases	46
1.4.11	Substrate binding domain	47
1.4.12	The crystal and three dimensional structure of $\alpha$ -L-AFases	48
1.5	Biotechnological applications of $\alpha$ -L-AFases	49
1.5.1	Production of arabinose as antiglycemic agent	49
1.5.2	Production of antimetastatic and anticarcinogenic	50
1.5.3	$\alpha$ -L-AFases and wine industry	51
1.5.3	$\alpha$ -L-AFases, acetic acid production and quality of	53
1.5.5	$\alpha$ -L-AFases in pulp and paper industry	54
1.5.6	$\alpha$ -L-AFases and animal feedstock	55
1.5.7	$\alpha$ -L-AFases in fruits juice industry	57
1.5.8	Production of fermentable sugars for bioethanol industry	58
1.5.9	Synthesis of pentose-containing compounds	60
1.6	Scope and Objectives of the present research	61

<b>CHAPTER 2</b>	<b>Isolation, Screening and Characterization of Marine Bacteria that Utilize Arabinose-Containing Substrates and Produce <math>\alpha</math>-L-Arabinofuranosidase.....</b>	<b>63</b>
	2.1 Introduction	63
	2.2 Materials and Methods	65
	2.3 Results and Discussion	71
<b>CHAPTER 3</b>	<b>Production, Isolation, Purification, Characterization and Substrate Specificity of <math>\alpha</math>-L-Arabinofuranosidase from <i>Pseudomonas</i> sp NIOCCAr27.....</b>	<b>90</b>
	3.1 Introduction	90
	3.2 Materials and Methods	92
	3.3 Results and Discussion	104
<b>CHAPTER 4</b>		
<b>SECTION 4.1</b>	<b>Optimization of Growth Medium and Conditions for <math>\alpha</math>-L-Arabinofuranosidase Production by <i>Bacillus</i> sp NIOCCW19 Grown under Solid State Fermentation and Submerged fermentation .....</b>	<b>130</b>
	4.1.1 Introduction	130
	4.1.2 Materials and Methods	131
	4.1.3 Results and Discussion	137
<b>SECTION 4.2</b>	<b>Isolation, Purification, Characterization and Substrate specificity of <math>\alpha</math>-L-Arabinofuranosidase Produced by <i>Bacillus</i> sp NIOCCW19 Grown under Solid-State Fermentation.....</b>	<b>160</b>
	4.2.1 Introduction	160
	4.2.2 Materials and Methods	161
	4.2.3 Results and Discussion	164

## **CHAPTER 5**

<b>SECTION 5.1</b>	<b>Cooperative Action and Application of <math>\alpha</math>-L-Arabinofuranosidase Produced by <i>Bacillus</i> sp NIOCCW19.....</b>	<b>189</b>
	5.1.1 Introduction	189
	5.1.2 Materials and Methods	190
	5.1.3 Results and Discussion	197
<b>SECTION 5.2</b>	<b>Comparative Evaluation of Catabolite Repression in Solid-State Fermentation and Submerged Fermentation for <math>\alpha</math>-L-Arabinofuranosidase and Xylanase Biosynthesis by <i>Bacillus</i> spNIOCCW19</b>	<b>213</b>
	5.2.1 Introduction	213
	5.2.2 Materials and Methods	214
	5.2.3 Results and Discussion	217
<b>CHAPTER 6</b>	<b>SUMMARY.....</b>	<b>232</b>
	<b>REFERENCES.....</b>	<b>242</b>
	<b>List of Publications.....</b>	<b>300</b>

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# *Chapter 1*

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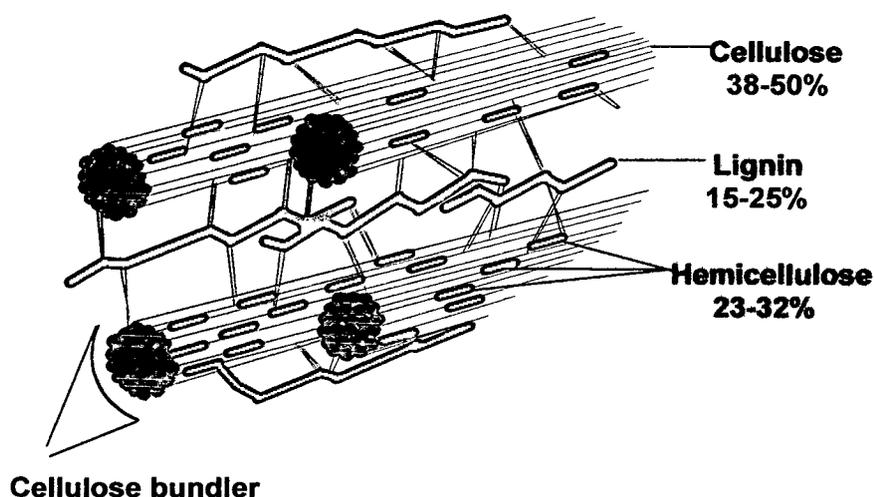
## Introduction and Literatures Review

### 1.1 INTRODUCTION

Lignocelluloses of plant cell walls are composed of cellulose, hemicellulose, pectin and lignin (Kanderperker and Numan, 2008). Hemicelluloses 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  $\alpha$ -L-arabinofuranosidases ( $\alpha$ -L-AFases) are accessory enzymes that cleave  $\alpha$ -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  $\alpha$ -L-AFase alone or in combination with other lignocellulose-degrading

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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 (Rémond *et al.*, 2002; Rémond *et al.*, 2004) and pretreatment of lignocelluloses for bioethanol production (Saha and Bothast, 1998b; Saha, 2003). Considering the potential and future prospects of  $\alpha$ -L-AFases, this literatures review deals with the various aspects of these enzymes with emphasis on their potential for biotechnology.



**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  $\beta$ -1,4-linked D-glucopyranose residuses which are thousands of units long. Cellobiose is the repeating unit in the cellulose molecule. Cellulose polymers are usually arranged to form crystalline microfibrils 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- $\beta$ -D-glucans and galactans. Xylans contain a  $\beta$ -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  $\alpha$ -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(O)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 Biloderis, 1995). A small proportion of oligomeric side-chains consisting of two or more arabinosyl residues linked via 1 $\rightarrow$ 2, 1 $\rightarrow$ 3 and 1 $\rightarrow$ 5 linkages has been reported although most arabinofuranosyl residues in arabinoxylans are found as monomeric substituents. Although arabinoxylans from various cereals and/or

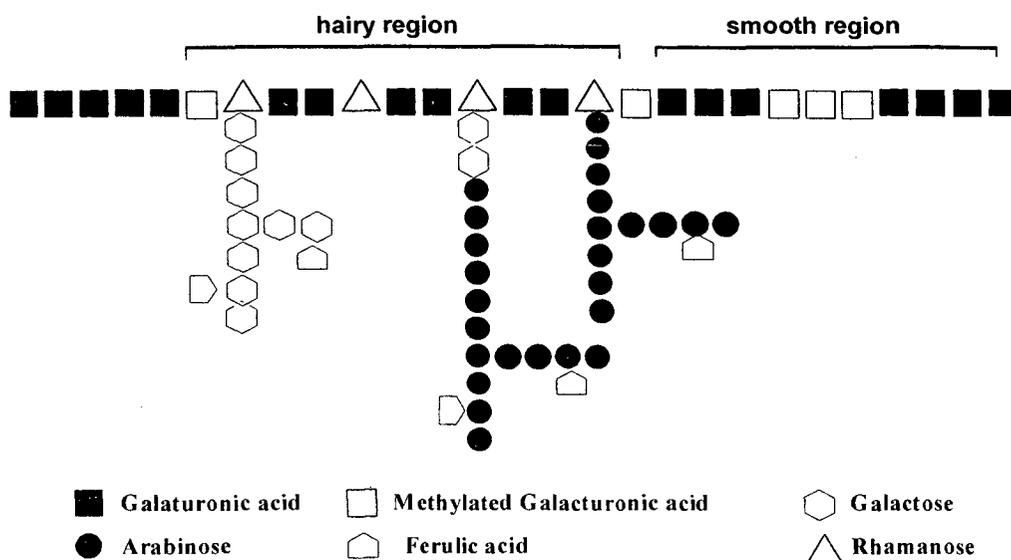


substituted or unsubstituted regions. It is also possible that two structurally distinct polymers exist. Molecular weight values reported for cereal arabinoxylans varied extensively depending on the fraction, source of the sample and the analysis method. Molecular weights ranging from about  $2 \times 10^4$  to  $1.7 \times 10^6$  were reported for cereal arabinoxylans (Fincher and Stone, 1986). Softwoods contain 10-15% arabino-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% anhydrouronic acid (Kormelink and Voragen, 1993). Rice bran neutral xylan contains 46% xylose, 44.9% arabinose, 6.1% galactose, 1.9% glucose, and 1.1% anhydrouronic 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.



**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, 1970a;



wood of maritime pine (*Pinus pinaster*) and the inner bark of *Rosa glauca* (Aspinall, 1970a,b; Joseleau *et al.*, 1977; Aspinall, 1980; Churms *et al.*, 1983 Voragen *et al.*, 1987).

#### **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- $\beta$ -D-glactan, arabino-1,3/6- $\beta$ -D-galactan and a related but a distinct group of cell wall glycoproteins containing arabinose and galactose. Arabinoglactan backbone is composed mainly of 1,3-linked D-galactopyranose units (Eriksson *et al.*, 1990). In all cases however, arabinoglactan backbone is usually substituted mainly at C-6 by single L-arabinose and/or D-galactose residues or longer branched arabinan or arabinoglactano-oligomers. Furthermore, considerable structural differences have been found between different members of the same arabinoglactan type (Clarke *et al.*, 1979; Aspinall, 1980).

Arabino-1,4- $\beta$ -D-glactans are present as pectic complexes in seeds, bulbs and leaves, and coniferous compression wood (van de Vis, 1994). Whereas, arabino-1,3/6- $\beta$ -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 arabinoglactans are also constituents of many exudate gums of angiosperms (e.g. *Acacia*) 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- $\beta$ -D-xylanases (EC 3.2.1.8) randomly attacking the backbone of  $\beta$ -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- $\beta$ -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  $\alpha$ -L-arabinofuranosidases. The  $\alpha$ -D-Glucuronidases (EC 3.2.1.131) hydrolyze the  $\alpha$ -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) liberating 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- $\alpha$ -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- $\alpha$ -L-arabinanases probably attack the  $\alpha$ -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,  $\alpha$ -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 $\alpha$ -L-arabinofuranosidases ( $\alpha$ -L-AFases)**

The  $\alpha$ -L-AFases ( $\alpha$ -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  $\alpha$ -L-AFases specifically catalyze the hydrolysis of terminal nonreducing- $\alpha$ -L-1,2-,  $\alpha$ -L-1,3-

and  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-arabinofuranosyl residues from various pectic, homo-hemicellulosic polysaccharides (branched arabinans, debranched arabinans), heteropolysaccharides (arabinogalactans, arabinoxylans, arabinoxyloglucans, glucuronoarabinoxylans, etc.) and different glycoconjugates is carried out by the  $\alpha$ -L-AFases (Beldman *et al.*, 1997; Sozzi *et al.*, 2002). Moreover, most microbial  $\alpha$ -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 $\alpha$ -L-AFases**

The importance of  $\alpha$ -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  $\alpha$ -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,  $\alpha$ -L-AFases possessing  $\beta$ -xylosidase activity or xylanases with  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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).  $\alpha$ -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  $\alpha$ -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,

$\beta$ -xylosidase and  $\alpha$ -L-AFase produced by *Aspergillus oryzae* HL15 during moromi fermentation. They also suggested that  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFases used in these experiments (Morales *et al.*, 1999).  $\alpha$ -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 $\alpha$ -L- arabinofuranosidase**

A bifunctional enzyme is an enzyme containing two distinct catalytic capacities in the same polypeptide chain. They usually catalyze two consecutive reactions

(Vrzheshch, 2007). 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 (Yournon *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 (*xln23*) 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- $\beta$ -D-xylopyranoside (*p*NPX). Several hemicellulases from different anaerobes, primarily ruminant bacteria, have been reported to have both  $\beta$ -xylosidase and  $\alpha$ -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 fibriosolvens* has been reported (Ult *et al.*, 1991). In *Bacteroides ovatus*, (*xsA*),  $\alpha$ -L-arabinofuranosidase and  $\beta$ -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*  $\beta$ 14 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/ $\beta$ -xylosidase which belongs to the family 54 of glycosyl hydrolases has a catalytic activity that hydrolyse the terminal non-reducing  $\alpha$ -L-arabinofuranoside residues in  $\alpha$ -L-arabinosides and 1,4- $\beta$ -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*  $\beta$ -xylosidase /

$\beta$ -xylosidase /  $\alpha$ -N-arabinofuranosidase has a 1.6-fold higher activity as an arabinosidase than as a  $\alpha$ -xylosidase when tested on the substrates nitrophenyl- $\beta$ -D-xylopyranoside and *p*NPA. Its catalytic activity is the hydrolysis of 1,4- $\beta$ -D-xylans so as to remove successive D-xylose residues from the non-reducing termini as well as hydrolysis of terminal non-reducing  $\alpha$ -L-arabinofuranoside residues in  $\alpha$ -L-arabinosides. This enzyme belongs to family 43 of glycosyl hydrolases (Ult *et al.*, 1991). *Streptomyces lividans*  $\alpha$ -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/ $\alpha$ -arabinosidases may show differences in their activity as  $\alpha$ -arabinosidases or xylosidases. The bifunctional xylosidase–arabinosidase (*xarB*) from thermophilic anaerobe *Thermoanaerobacter ethanolicus* JW200 showed different temperature optima for  $\alpha$ -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 $\alpha$ -L-AFases

The regulation mechanism of the biosynthesis of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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 Ara E permease and enters the pathway after conversion to D-xylulose and D-Xylulose 5-phosphate by D-xylose isomerase and D-xylulose kinase, respectively (Gärtner *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  $\alpha$ -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 araABDLMNPQ-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  $\alpha$ -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*  $\beta$ 14 (Gasparic *et al.*, 1995a); *Aspergillus niger* (Ruijter *et al.*, 1997); *Aspergillus nidulans* (Gielkens *et al.*, 1999) and *Penicillium purpurogenum* (De Ioannes *et al.*, 2000; Carvalho *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  $\alpha$ -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  $\alpha$ -L-AFase in SSF are not available.

#### **1.4.4 Microbial production of $\alpha$ -L-AFases**

The  $\alpha$ -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  $\alpha$ -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 cellobiose, lactose, lactulose, maltose, mellibiose, sucrose, trisaccharide, raffinose, D-arabitol, L-arabitol, 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  $\alpha$ -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  $\beta$ -1,4-galactan, carob galactomannan, Me- $\beta$ -xyloside and lactobionic acid are some other carbon sources utilized for  $\alpha$ -L-AFases production.

Generally, arabinose-containing substrates are essential for the efficient production of  $\alpha$ -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  $\alpha$ -L-AFases (Beylot *et al.*, 2001a;b; Koseki *et al.*, 2003). Arabinogalactans and oatmeal were found to be the best inducers for  $\alpha$ -L-AFase isolated from *Bacillus pumilus* PS213 (Degrassi *et al.*, 2003). *Rhodothermus marinus* produced  $\alpha$ -L-AFase when grown on birchwood xylan (Gomes *et al.*, 2000). L-Arabitol induced production of  $\alpha$ -L-AFases enzymes araA and araB by the *A. niger* mutants (de Grootet *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  $\alpha$ -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  $\alpha$ -L-AFase, followed by arabitol. However, oat spelt xylan, sugar beet arabinan, xylose, xylitol, and wheat arabinoxylan were intermediate in their ability to support the  $\alpha$ -L-AFase production (Saha and Bothast, 1998a). In the presence of arabitol, *Aspergillus terreus* CECT 2663 produced three  $\alpha$ -L-AFases (Le Clinche *et al.*, 1997). The filamentous fungus *Cochliobolus carbonum* produced  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFase (4.2 U/ml) by

Lachke, 1995). Formation of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFase by *Streptomyces diastaticus* (Tajana *et al.*, 1992). Arabinan, as the carbon source, was the most effective substrate for the production of  $\alpha$ -L-AFase by *Corticium rolfsii* (Kaji and Yoshihara, 1970). Arabinan-containing carbon sources have efficiently induced  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFase by *A. nidulans* was high when ammonium sulfate or ammonium chloride was used as nitrogen source (Fernández-Espinar *et al.*, 1994). *Aspergillus kawachii* IF04308 produced highest amounts of  $\alpha$ -L-AFase when grown on a medium supplemented with a mixture of bacto-tryptone, yeast extract and NaNO<sub>3</sub> 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  $\alpha$ -L-AFase by *Penicillium purpurogenum* was enhanced (De Ioannes *et al.*, 2000). The optimal medium composition for  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFases production by bacteria (Gomes *et al.*, 2000; Degraasi *et al.*, 2003) and fungi (Jankiewicz and Michniewicz, 1987; Hashimoto and Nakata, 2003; Sakamoto and Kawasaki, 2003). Yields of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFases production by bacteria using SSF.

#### **1.4.5 Biochemical properties of $\alpha$ -L-AFases**

Microbial  $\alpha$ -L-AFases vary in their molecular masses, which can be as high as 495 kDa for  $\alpha$ -L-AFase from *Streptomyces purpurascens* IFO3389 (Komae *et al.*, 1982) (Table 1.1). Multiple forms of  $\alpha$ -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. purpurogenum* and *Sclerotinia fructigena* (Laborda *et al.*, 1973). In contrast, few bacteria produce multiple forms of  $\alpha$ -L-AFase including *Streptomyces diastaticus* (Tajana *et al.*, 1992).

**Table 1.1: Microbial  $\alpha$ -L-arabinofuranosidases: some biochemical characteristics and substrate specificity**

Microbial source	Mw kDa	Opt. pH	Opt. Temp.°C	$K_m$ mM	$V_{max}$ U/mg	Active on Substrates	References
<b>Bacteria</b>							
<i>Bcillus subtilis</i> .	65	6.5	ND	ND	ND	AOS,BA, , not active on AX	Weinstein and Albersheim, (1979)
<i>Bcillus Stearothermophilus L1</i>	110	7	70	0.22	110	AX only	Bezalel <i>et al.</i> , (1993)
<i>Bcillus subtilis 3-6</i>	61	7	60	ND	ND	AOS, BA, not active on AX, AG	Kaneko <i>et al.</i> , (1994)
<i>Bcillus polymyxa</i>	166	6.5	50	0.324	214.1	AOS, not active DA, AX, AG	Morales <i>et al.</i> , (1995)
<i>Bcillus stearothermophilus T-6</i>	256	5.5-6	70	0.42	749	BA , low activity on OSX	Gilead and Shoham, (1995)
<i>Thermobacillus xylanilyticus</i>	56.1	5.6 - 6.2	75	0.5	555	AX,OSX	Debeche <i>et al.</i> , (2000)
<i>Bcillus pumilus</i>	220 60	7	55	1.3	52.9	ND	Degrassi <i>et al.</i> , (2003)
<i>Bcillus stearothermophilus</i> No. 236	190	6.5	55	1.19	26.1	AX, not active on OXS	Kim <i>et al.</i> , (2004)
<i>Geobacillus caldoxylolyticus TK4</i>	236	6.0	75-80	0.17	588.2	AOS,BA, not active on AX,AG	Canakci <i>et al.</i> , (2007)
<i>Bacillus pumilus</i> ARA	56 140 <sup>a</sup>	6.4	60	1.05	240	OSX	Pei and Shao, (2008)
<i>Clostridium cellulovorans</i>	138	6	40-50	0.71	75.8	BA, AX	Kosugi <i>et al.</i> , (2002)
<i>Clostridium thermocellum</i>	-	7	82	0.25	NR	BA, AX, AX2, A2	Taylor <i>et al.</i> , (2006)
<i>Clostridium acetobutylicum</i> ATCC824	94	5.0-5.5	-	-	-	BA, pNPGI, oligosaccharides from OSX and AX	Lee and Forsberg, (1987)

Table 1.1 Cont.

Bacteria	Mw kDa	Opt. pH	Opt. Temp.°C	$K_m$ mM	$V_{max}$ U/mg	Active on Substrates	References
<i>Clostridium stercorarium</i>	53.3	7	-	-	-	AX	Sakka <i>et al.</i> , (1993)
<i>Clostridium stercorarium</i>	195	5	-	-	-	Oat Spelt arabinoxylan, AG	Schwarz <i>et al.</i> , (1995)
<i>Bifidobacterium breve</i> K-110	60	4.5	45	0.22	9.3	Ginsenoside Rb2 and Rc	Shin <i>et al.</i> , (2003)
<i>Bifidobacterium longum</i> B667	61 ° 260 <sup>a</sup>	6.0	45	0.295	417	BA, AX, A2, A3, A4, A5	Margolles Reyes-Gavilán, (2003)
<i>Bifidobacterium adolescents</i> DSM 20083	100	6.0	30–40	NR	NR	Wheat flour AX only	Van Laere <i>et al.</i> , (1997); Piston <i>et al.</i> , (1996)
<i>Butyrivibrio fibrisolvens</i> GS113	240	6.0–6.5	45	0.7	109	BA, AX, OSX	Hespell and O'Bryan, (1992)
<i>Ruminococcus albus</i>	310	6.9	37	1.3	NR	Alfalfa cell wall AX	Greve <i>et al.</i> , (1984)
<i>Bacteroides xylanolyticus</i>	364	5.5–6.0	50	0.5	155	AXO only	Schyns <i>et al.</i> , (1994)
<i>Cytophaga xylanolytica</i> XM3 (DSM 6779)	57.7 59.2	5.8 NR	45 NR	0.504 NR	319 NR	BA, rye, wheat, corn cob and oat spelt AXs, BA	Renner and Breznak, (1998); Kim <i>et al.</i> , (1998)
<i>Erwinia carotovora</i> IAM 1024	-	6.0	-	-	-	BA only, not pNPAF, AX, AG, DA	Kaji and Shimikawa, (1984)
<i>Pseudomona cellulosa</i>	57	5.5	<55	0.18	NR	WA, BA, A2, A3	Beylot <i>et al.</i> , (2001)
<i>Thermoanaerobactere ethanolicus</i> JW200	85	NR	65	NR	NR	-	Mai <i>et al.</i> , (2000)
<i>Thermotoga maritima</i> MSB8	332 55.3	7	90	0.416	NR	BA, DA	Miyazaki, (2005)
Bacterium PRI-1686	350	6	70	0.6	122	BA, DA, OSX	Birgisson <i>et al.</i> , (2004)

Table 1.1 Cont.

Microbial source	Mw kDa	Opt. pH	Opt. Temp.°C	$K_m$ mM	$V_{max}$ U/mg	Active on Substrates	References
<b>Actinomycetes</b>							
<i>Streptomyces chartreusis</i> GS901	80	5.5	55	-	-	BA, AX, AG, A2, A3	Matuso <i>et al.</i> , (2000)
	37	7	50	-	-	BA, AX, AG, A2	
<i>Streptomyces thermaviolaces</i> OPC-520	37	5	60	-	-	AX, OSX	Tsujibo <i>et al.</i> , (2002)
<i>Streptomyces</i> sp. 17-1	92	6.0	-	-	-	BA, DBA, AX, AG	Kaji <i>et al.</i> , (1981)
<i>Streptomyces diastaticus</i>	38	4.0-7.0	50	10	0.4	AX, DA	Tajana <i>et al.</i> , (1992)
	60	4.0-7.0	50	12.5	1.25	AX, DA	
<i>Streptomyces diastatochromogenes</i>	73	6.0	-	-	-	BA,	Higashi <i>et al.</i> , (1983)
<i>Streptomyces purpurascens</i> IFO 3389	495	6.5	-	-	-	A2, A3	Komae <i>et al.</i> , (1982)
<i>Streptomyces lividans</i> 66 strain 1326	380 69	6	60	0.6	180	Arabinoxyloligosaccharides Low on BA, DBA, rye, wheat flour AXs,	Manin <i>et al.</i> , (1994)
<i>Streptomyces lividans</i> IAF10-164 ( <i>msiK</i> )	43	6.0	-	-	-	rye, wheat and oat spelt AXs	Vincent <i>et al.</i> , (1997)
<i>Thermomonospora fusca</i> BD21	92	-	-	-	-	OSX oligosaccharides	Bachmann and McCarthy, (1991)
<i>Thermomonospora fusca</i>	~92	9	65	0.18	0.04	-	Tuncer, (2000); Tuncer and Ball, (2003b)

**Table 1.1 Cont.**

Microbial source	Mw kDa	Opt. pH	Opt. Temp.°C	$K_m$ mM	$V_{max}$ U/mg	Active on Substrates	References
<b>Fungi</b>							
<i>Aspergillus oryzae</i> HL15	60	5.5	60	-	-	AX, AG, OSX	Hashimoto and Nakata, (2003)
<i>Aspergillus oryzae</i>	228 55	5	50	-	-	-	Matsumura <i>et al.</i> , (2004)
<i>Aspergillus Kawachii</i>	80 62	4 4	55 55	-	-	AX AX	Koseki <i>et al.</i> , (2003)
<i>Aspergillus awamori</i> IFO 4033	81 32	4.0 4.0	60 60	-	-	BA,DA,wheat AX, AG, gum arabic (both enzyme)	Kaneko <i>et al.</i> , (1998a)
<i>Aspergillus nidulans</i> CECT 2663	65	4.0	65	0.679	9.94	-	Ramon <i>et al.</i> , (1993)
<i>Aspergillus niger</i> 5-16	67 53	4.0 4.0	60 -	-	-	BA BA, AX	Kaji and Tagawa, (1970)
<i>Aspergillus sojae</i>	34.3	5.0	50	-	-	AX,BA,AG	Kimura <i>et al.</i> , (1995)
<i>Aspergillus terreus</i>	39 59 59	3.5-4.5 3.5-4.5 3.5-4.5	-	0.43 0.65 0.76	146 295 274	OSX,RAX, AGX, BA OSX,RAX, AGX, BA RAX, AGX, BA	Luonteri <i>et al.</i> , (1995)
<i>Aspergillus niger</i>	83	3.4	-	-	-	Debranched arabinooligosaccharides only	Romboust <i>et al.</i> , (1988);van der Veen <i>et al.</i> , (1991);
<i>Aspergillus nidulans</i>	36	5.5	55	2.7	-	pNPAF	Fernández-Espinar <i>et al.</i> , (1994)
<i>Aspergillus aculeatus</i>	37 37	3.0-3.5 4.0-4.5	-	-	-	SBA, linear apple Arabinan,	Beldman <i>et al.</i> , (1993)

Table 1.1 Cont.

Microbial source	Mw kDa	Opt. pH	Opt. Temp.°C	$K_m$ mM	$V_{max}$ U/mg	Active on Substrates	References
<i>Aspergillus niger</i>	61	3.7-0.4	-	-	-	Monoterpenyl arabinofuranosyl-glucosides	Gunata <i>et al.</i> , (1990)
<i>Aspergillus awamori</i> (CMI)142717 IMI	64	4.6	50	1.39	-	BA, larchwood AG, wheat straw AX(water soluble), OSX, oat straw, barley straw, rye grass, Timothy grass and larchwood arabinoxylan	Wood and McCrea (1996); Piston <i>et al.</i> (1996)
<i>Aspergillus awamori</i> (CMI)142717 IMI	32	5	-	-	-	Wheat and oat spelts AX	Kormelink <i>et al.</i> (1991); Kormelink <i>et al.</i> , (1993); Piston <i>et al.</i> , (1996)
<i>Aspergillus niger</i>	97	5	50	-	-	Citrus pectin, AX, BA	Muzakhar, (2002)
<i>Aspergillus niger</i> K1	53	3.8-4.0	30	0.5	-	BA, DA, AX, gum arabic	Kaji <i>et al.</i> , (1969) Kaji and Tagawa (1970); Tagawa and Kaji, (1988)
<i>Aspergillus niger</i> N400 (CBS12049)	83 67	3.4 3.8	-	-	-	pNPAF pNPAF, BA	Flipphi <i>et al.</i> (1993a); Flipphi <i>et al.</i> (1993b)
<i>Aspergillus terreus</i> CECT 2663	90 82 78.5	5.5 5 5	50 60 65	0.35 0.47 0.31	133 175 75	- - -	Le Clinche <i>et al.</i> (1997)

1.1  
 Table 3.5 Cont  
 9/5/09

Microbial source	Mw kDa	Opt. pH	Opt. Temp.°C	$K_m$ mM	$V_{max}$ U/mg	Active on Substrates	References
<i>Penicillium purpurogenum</i>	49.6	5	50	1.23	-	AX, BX, OSX, IAG, WS, WB	De Ioannes <i>et al.</i> , (2000); Carvallo <i>et al.</i> , (2003)
<i>Penicillium chrysogenum</i>	79	4	50	0.86	-	BA,DA,AX, AG,AOs	Sakamoto and Kawasaki, (2003)
	52	3.3-5.0	50	1.32	-		
<i>Penicillium capsulatum</i>	60 000	3.5	60	0.18	34.3	BA,AX, AXO	Filho <i>et al.</i> , (1996)
	64 000	4.0	60	1.3	80.4	BA, AX, AXO	
<i>Penicillium purpurogenum</i>	70	5	60	0.098	-	AOs, low activity against AX, BA, DA	Fritz <i>et al.</i> , (2008)
<i>Trichoderma reesei</i>	35	4	60	1.1	304	OSX	Nogawa <i>et al.</i> , (1999)
	53	4	60	0.9	444		
<i>Trichoderma reesei</i>	53	4.0	-	-	-	BA, AXO	Poutanen, (1988a)
<i>Trichoderma reesei</i>	53	4.5	-	-	-	BA, wheat, wheat straw, oat spelt, rye AXs, pin kraft pulp arabinoglucuronoxylan	Poutanen, (1988b); Margolles-Clark <i>et al.</i> , (1996)
<i>Fusarium oxysporum f. sp. dianthi</i>	58	4.0	50°C	1.48	-	OSX	Chacón-Marténez <i>et al.</i> , (2004)
<i>Fusarium oxysporum</i>	200	6	50-60	0.39	1.6	Arabinan	Panagiotou <i>et al.</i> , (2003)
	180	6	50-60	0.29	4.6		
<i>Sclerotinia sclerotiorum</i>	63 000	4.0-4.5	-	-	-	BA, AX,AG	Baker <i>et al.</i> , (1979)
<i>Sclerotinia fructigena</i>	-	4.0	-	-	-	BA, apple fiber	Fielding and Byrde, (1969)
<i>Phytophthora palmivora</i> (Butl.) Butl	63.1	4	50	0.65	-	-	Akinrefon, (1968)

1-1  
 Table 35 Cont 8/5/09

Microbial source	Mw kDa	Opt. pH	Opt. Temp.°C	$K_m$ mM	$V_{max}$ U/mg	Active on Substrates	References
<i>Rhizomucor pusillus</i> HHT1	88	4.0	65	0.59	387	BA, A2, A3, A4, A5	Rahman <i>et al.</i> , (2001); (2003)
<i>Corticium rolfsii</i>	-	2.5	30	-	-	AX,BA,DA	Kaji and Yoshihara, (1971)
<i>Pichia capsulate</i> X91	250 72	6	50	-	-	AOs,BA,AG, Monoterpenyl arabinofuranosylglucosides	Yannai and Sato, (2000)
<i>Humicola insolens</i>	-	6.7	53	-	-	Wheat AX, mono and double substituted AX	Sørensen <i>et al.</i> , (2006a)
<i>Aureobasidium pullulans</i>	210	4.0–4.5	75	0.26	6.99	BA, AX, OSX	Saha and Bothast, (1998a;b)
<i>Rhodotorula flava</i>	-	2	50	9.1	72.5	BA, DA, AX	Uesaka <i>et al.</i> , (1978)
<i>Termitomyces clypeatus</i>	-	5.5	-	-	-	AX,BA,DA,AG, arabinoxylan oligosaccharides	Sinha and Sengupta, (1995)
<i>Cochliobolus carbonum</i>	63	3.5–4.0	50	-	-	BA, AX	Ransom and Walton, (1997)
<i>Dichomitus squalens</i>	60	3.5	60	1.64	-	BA, AX	Brillouet <i>et al.</i> , (1985)

A2:arabinobiose, A3: arabinotriose, A4:arabinotetraose, A5: arabinopentaose, AOS: arabinooligosaccharides, BA:branched Arabinan, AX: arabinoxylan, AG: arabinogalactan, DA: debranched Arabinan, OSX: oat septil xylan, , PNPGI: p-nitrophenyl-β-D-galactoside, WA: wheat arabinoxylan, PNPAF: p-nitrophenyl-α-L-arabinofuranoside, RAX:ray arabinoxylan, AGX:arabinogalactan-xylooligosaccharides; SBA:sugar beet Arabinan, IAG:Irichwood arabinogalactan, WB:wheat bran, WS: wheat straw, AXO:arabinoxylooligosaccharides,ND:not determined.

The effect of temperature and pH on the  $\alpha$ -L-AFase depends on the source from which the enzyme is isolated (**Table 1.1**). The highest temperature stability has been obtained for  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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; Bezalet *et al.*, 1993; Fernández-Espinar *et al.*, 1994; Filho *et al.*, 1996; Saha and Bothast, 1998b). However, reported fungal  $\alpha$ -L-AFase tend to be more acidophilic than bacterial  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFase (abfB) from *Bifidobacterium longum* B667 (Margolles and Reyes-Gavilán, 2003) and

$\alpha$ -L-AFase (AbfD3) from *Thermobacillus xylanilyticus* D3 (Debeche *et al.*, 2000) were not affected by EDTA, DTT, but were affected by  $\text{Cu}^{2+}$  ions. Metal ions such as  $\text{Ag}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$  and Ni had an inhibitory effect on some of these enzymes (Tsujiho *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  $\alpha$ -L-arabinofuranosidases or  $\alpha$ -arabinosidases (EC 3.2.1.55) and endo acting 1 $\rightarrow$ 5- $\alpha$ -L-arabinan 1 $\rightarrow$ 5- $\alpha$ -L-arabinanohydrolases (EC 3.2.1.99), more commonly called endo 1 $\rightarrow$ 5- $\alpha$ -L-arabinanases.

##### **1.4.6.2 Kaji (1984) classification**

Kaji (1984) subdivided  $\alpha$ -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  $\alpha$ -L-arabinofuranosidases act only on low molecular weight substrates, such as *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (*p*NPAF) and L-arabino-oligosaccharides, and are inactive against polymers. Type B  $\alpha$ -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- $\alpha$ -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- $\alpha$ -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 $\rightarrow$ 4)-[ $\beta$ -D-arabinoxylan] arabinofuranohydrolase), are specifically active on arabinofuranosidic linkages in arabinoxylans from oat spelt, wheat or barley, and have no activity toward *p*NPA (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 *p*NPAF 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)  $\alpha$ -L-arabinofuranosidases (E.C 3.2.1.55) that are not active toward polymers, and show activity against  $\alpha$ -1,5-L-arabinofuranooligosaccharides

oligomers from arabinan , arabinoxylan , arabinogalactan as well as *p*NPAF (Weinstein and Albersheim,1979; Komae *et al.*, 1982), (2)  $\alpha$ -L-arabinofuranosidases which are active toward polymers as well as oligomers from arabinan, arabinoxylan and arabinogalactan and *p*NPAF (Kaji and Tagawa, 1970; Rombouts *et al.*, 1988), (3)  $\alpha$ -L-arabinofuranohydrolases specific for arabinoxylan from oat spelts, barley and wheat but show no activity against *p*NPAF (Kormelink *et al.*, 1991; Van Laere *et al.*, 1997), (4) Exo- $\alpha$ -L-arabinanases which are not active toward *p*NPAF but act in exo manner on the side chains of branched arabinan, (5)  $\beta$ -L-arabinopyranosidases which are active only against *p*-nitrophenyl- $\beta$ -L-arabinopyranoside, and (6) Endo-1 $\rightarrow$ 5- $\alpha$ -L-arabinanase (E.C.3.2.1.99) which are active in degrading linear 1 $\rightarrow$ 5- $\alpha$ -L-arabinan.

#### 1.4.6.4 Numan and Bhosle (2006) classification

Kaji (1984) classified  $\alpha$ -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- $\alpha$ -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  $\alpha$ -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  $\alpha$ -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- $\alpha$ -L-arabinofuranoside. This subclass was represented by the enzyme isolated from *B. adolescentis* (Van Laere *et al.*, 1999).

Recently, new types of  $\alpha$ -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  $\alpha$ -1,5 backbone and  $\alpha$ -1,3-side chains of arabinan and debranched arabinans. In addition, they are able to act on *p*-nitrophenyl  $\alpha$ -L-arabinofuranoside and some were also able to act on *p*-nitrophenyl  $\beta$ -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  $\alpha$ -L-arabinofuranosidase. The enzymes isolated from the thermophilic bacterium PRI-1686 (Birgisson *et al.*, 2004) and from the hyperthermophilic bacterium *Thermotoga maritima* MSB8 represent these subclasses (Miyazaki, 2005) (Table 1.2).

**Table 1.2:** Classification of arabinose-releasing enzymes based on Numan and Bhosle (2006).

Enzyme Class	Enzyme subclass	pNPAP	pNPAF	Arabinan		Arabinogalactan		Arabinoxylan	
				O	P	O	P	O	P
Type A $\alpha$ -L-Arabinofuranosidase	$\alpha$ -L-Arabinofuranosidase	-	+	+	-	+	-	+	-
Type B $\alpha$ -L-Arabinofuranosidase	$\alpha$ -L-Arabinofuranosidase	-	+	+	+	+	+	+	+
Type C $\alpha$ -L-Arabinofuranosidase	Tm-AFase	-	+	+	+	-	-	+	+
	TB-AFase	+	+	+	+	-	-	-	-
$\alpha$ -L-Arabinofuranohydrolase	AXH m,d (2,3)	-	-	-	-	-	-	+	+
	AXHm (2,3)	-	-	-	-	-	-	+	+
	AXHd (C3)	-	-	-	-	-	-	+	+
$\beta$ -L-Arabinopyranosidase	$\beta$ -L-Arabinopyranosidase	+	-	-	-	-	-	-	-
Exo- $\alpha$ -L-arabinanase	Exo- $\alpha$ -L-arabinanase	-	-	-	+	-	-	-	-
Endo- $\alpha$ -L-arabinanase	Endo- $\alpha$ -L-arabinanase	-	-	+	+	-	-	-	-

pNPAP : *p*-nitrophenyl- $\beta$ -L-arabinopyranoside ; p-NPAF: *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside;

O: Oligomeric; P: polymeric

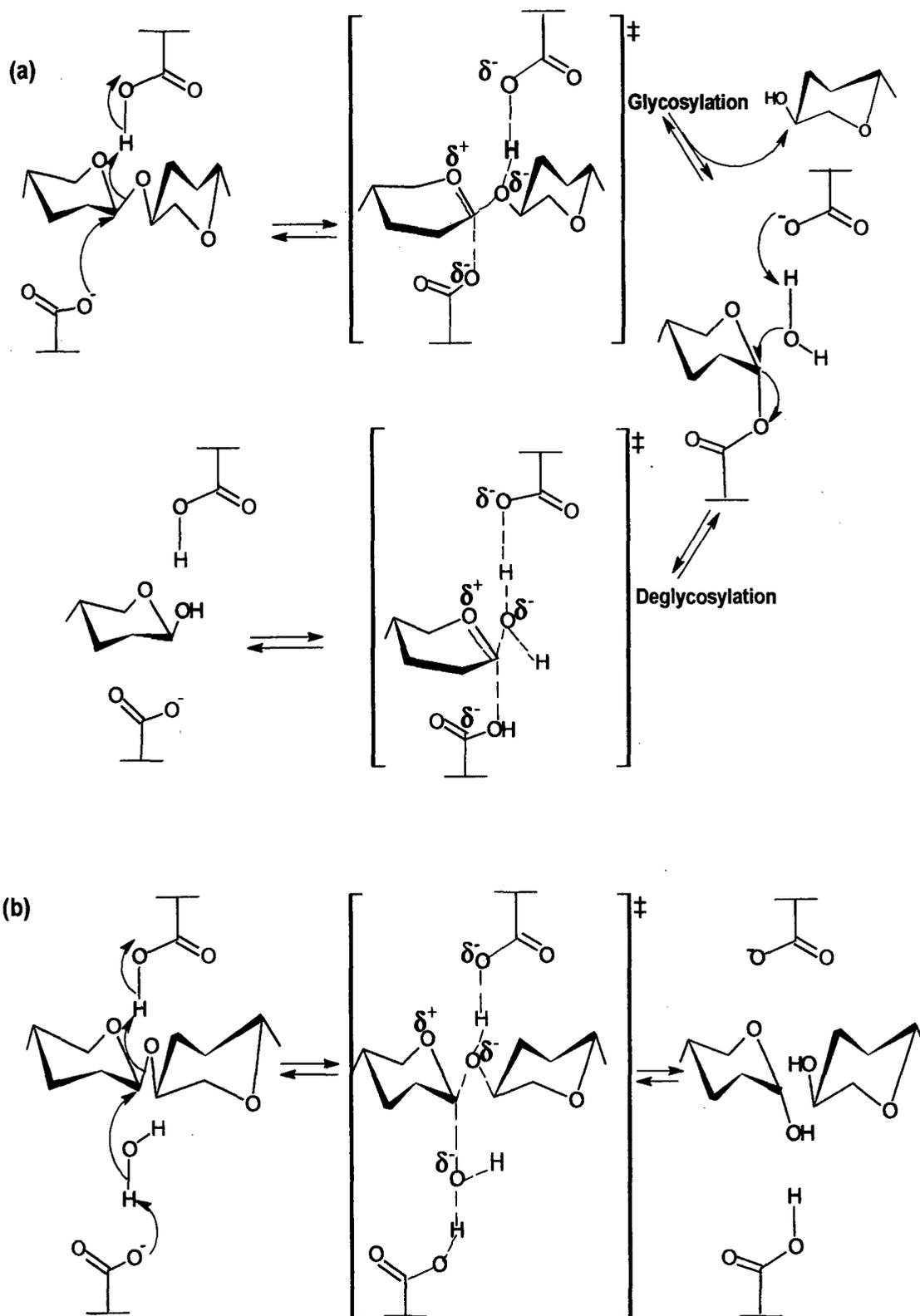
#### 1.4.6.5 Classification at molecular level

The most recent classification scheme based on amino acid sequences, primary structure similarities and hydrophobic cluster analysis has classified  $\alpha$ -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 $\alpha$ -L-AFases

Like other glycoside hydrolases,  $\alpha$ -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  $\alpha$ -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 Hövel *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  $\alpha$ -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 $\alpha$ -L-AFases**

To date, there are more than 110 amino acid sequences of different  $\alpha$ -L-AFase. Based on amino acid sequence homology,  $\alpha$ -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 specifics 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  $\alpha$ -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  $\alpha$ -L-AFases classified as GH51 glycoside hydrolases, (Coulinho and Henrisst, 2000; Shallom *et al.*, 2002b). Studies on family 51  $\alpha$ -L-AFases, from *Thermobacillus xylanilyticus* (AbfD3) have led to the localization of its two catalytic residues. These are Glu<sup>176</sup> that represents the acid-base catalyst and Glu<sup>298</sup> that represents the nucleophile (Debeche *et al.*, 2000; Debeche *et al.*, 2002). In contrast, the catalytic residues of family 51  $\alpha$ -L-AFase from *Geobacillus stearothermophilus* T-6 (AbfA) were recently identified as Glu<sup>175</sup> (the acid-base catalyst) and Glu<sup>294</sup> (the nucleophile) (Shallom *et al.*, 2002a; b; Hövel *et al.*, 2003). As family 51  $\alpha$ -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  $(\beta/\alpha)_8$  scaffold, modify the overall active structure. Another study on (AbfD3) revealed a third glutamate (Glu<sup>28</sup>) which is not directly involved in catalysis. Nevertheless, Glu<sup>28</sup> is critical for normal hydrolytic activity as it might be involved in the stabilization of a catalytic transition state. The Glu<sup>28</sup> 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<sup>28</sup> 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<sup>380</sup> and Glu<sup>467</sup>. Additionally they have unknown functional domains

existing in the N-terminal and C-terminal regions (Matsuo *et al.*, 2000). Glu<sup>194</sup> and Glu<sup>321</sup> 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 arabinoxylans 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  $\alpha$ -L-arabinofuranosidases/ $\beta$ -xylosidases (EC 3.2.1.37) able to hydrolze terminal non- reducing D-xylose, but also  $\alpha$ -L-arabinofuranose residues from  $\alpha$ -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 $\alpha$ -L-AFases**

Microorganisms produce  $\alpha$ -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  $\alpha$ -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 from *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 pNP- $\alpha$ -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 thermoviolaceus* expresses (STX-IV) a gene coded for  $\alpha$ -L-AFase which exhibited similar substrates specificity to that of enzymes from *Streptomyces lividans*, *A. awamori* and *Clostridium stercorarium*. These enzymes preferred arabinoxylan but not other arabinosaccharides and showed a low activity with pNPAF (Tsujiibo *et al.*, 2002). The  $\alpha$ -L-AFase I from *A. awamori* preferentially hydrolyzed the (1 $\rightarrow$ 5) linkage of branched arabinotrisaccharide, whereas  $\alpha$ -L-AFase II from the same organism preferentially hydrolyzed (1 $\rightarrow$ 3) linkage in the same substrate (Kaneko *et al.*, 1998a). The  $\alpha$ -L-AFase I released arabinose from the nonreducing terminus of arabinan, whereas  $\alpha$ -L-AFase II preferentially hydrolyzed the arabinosyl side chain linkage of arabinan. A novel property of  $\alpha$ -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 $\alpha$ -L-AFases**

Some  $\alpha$ -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 thermoviolaceus* OPC-520 chromosome (Tsujiibo *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),  $\alpha$ -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* (Carvalho *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  $\alpha$ -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 stercoarium* (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. xylanolyticus* (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. stercoarium xylA* gene encoding a bifunctional protein with  $\beta$ -D-xylosidase and  $\alpha$ -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  $\alpha$ -L-AFases in the release of arabinofuranosyl residues is not yet clear (Kimura *et al.*, 2000). Some  $\alpha$ -L-AFases with SBD have been reported, i.e.,  $\alpha$ -L-AFase from *Streptomyces lividans* has a cellulose-binding domain (CBD) (Vincent *et al.*, 1997). Other  $\alpha$ -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  $\alpha$ -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*  $\alpha$ -L-AFase (Abf62A) binds to polysaccharides. This protein comprises a new family of CBMs, designated as Abf62A-CBM35. There are more than 13  $\alpha$ -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 $\alpha$ -L-arabinofuranosidase**

So far, only three  $\alpha$ -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  $\alpha$ -L-AFase B (AkabfB) (EC 3.2.1.55) from *A. kawachii* IFO 4308 located within GH 54 family (Miyanaga *et al.*, 2004),  $\alpha$ -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/  $\alpha$ -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 $\alpha$ -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.  $\alpha$ -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.

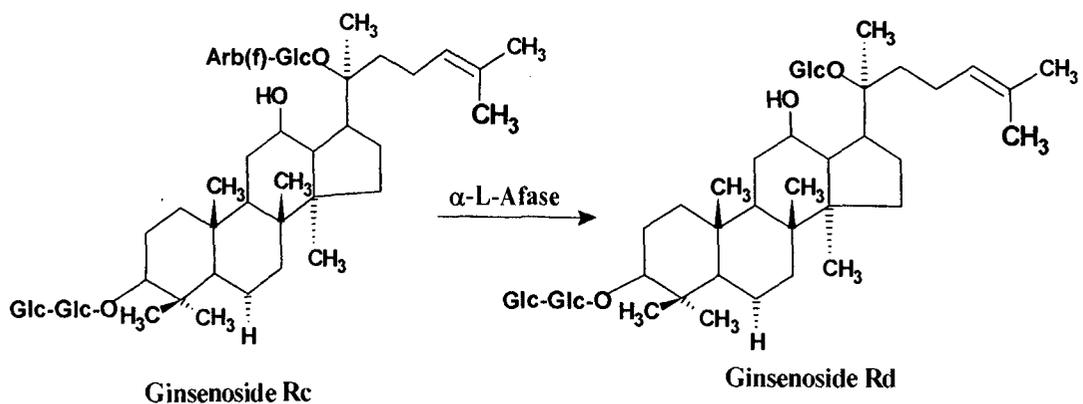
#### **1.5.1 Production of arabinose as antiglycemic agent**

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 prerequisite 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  $\alpha$ -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  $\alpha$ -L-AFase (Bae *et al.*, 2000) (Fig. 1.6).



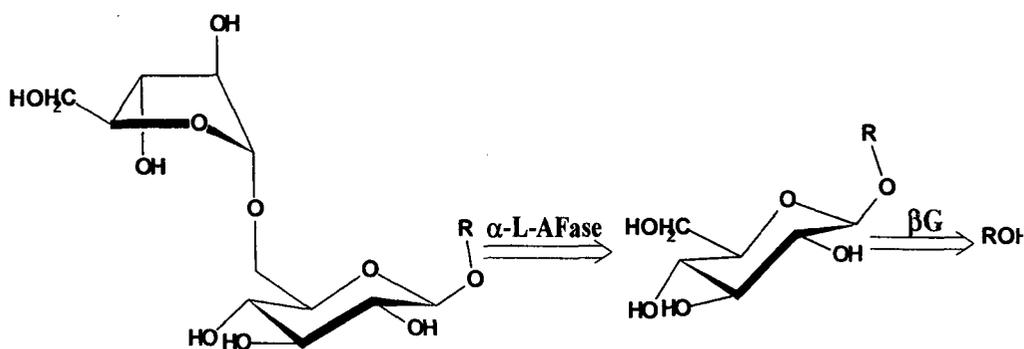
**Fig. 1.6:** Proposed metabolic conversions for the ginsenoside Rb2 by  $\alpha$ -L-arabinofuranosidase from *B. breve* K-110. Modified from Shin *et al.* (2003).

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  $\alpha$ -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  $\alpha$ -L-AFases and  $\alpha$ -L-arabinopyranosidase (Kaji and Tagawa, 1970).

### **1.5.3 $\alpha$ -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 Jimenez, 2000). Terpenols are strongly aromatic molecules that represent an important part of aromas (Giinata *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  $\alpha$ -L-arabinofuranose which can be released by the action of  $\alpha$ -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  $\alpha$ -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).



**Fig. 1.7:** Mechanism of action of the glycosidase  $\alpha$ -L-arabinofuranosidase and  $\beta$ -D-glucopyranosidase ( $\beta$ G) on diglycosidic precursors. ROH is a volatile aglycone such as monoterpenols and other alcohols. Modified from Spagna *et al.* (1998).

Thus,  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFase,  $\beta$ -D-glucopyranosidase and  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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 ( $\alpha$ -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,  $\alpha$ -L-arabinofuranosidase and  $\beta$ -Dglucopyranosidase ( $\beta$ G, 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 $\alpha$ -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  $\alpha$ -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,  $\alpha$ -L-AFase from *A. niger* and *Lactobacillus plantarum* 20B, soluble carbohydrate availability, acidification rate and production of acetic acid increased during sourdough fermentation.  $\alpha$ -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,  $\alpha$ -L-AFases along with pentosanase 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 $\alpha$ -L-AFases in pulp and paper industry**

Several commercial xylanase preparations are available for the treatment of pulp (Viikari *et al.*, 1994). Application of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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 NaClO<sub>2</sub> 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  $\alpha$ -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  $\alpha$ -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 $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFase from *P. ruminicola*  $\beta$ 14 was used (Gasparic *et al.*, 1995a;b).

### **1.5.7 $\alpha$ -L-AFases in fruits juice industry**

$\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFase and endo-arabinanase (Whitaker, 1984; Mc Cleary *et al.*, 1988; Voragen *et al.*, 1988). Birgisson *et al.* (2004) reported an  $\alpha$ -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  $\alpha$ -1,5 backbone and  $\alpha$ -1,3-side chains of arabinan. Moreover, Miyazaki (2005) described a thermophilic  $\alpha$ -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,

2003; Sørensen *et al.*, 2005). 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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFases are robust and thermostable and do not require the use of costly sugar donors. For example, thermostable  $\alpha$ -L-AFase (AbfD3) from *T. xylanilyticus* (Debeche *et al.*, 2000) has the ability to catalyze transglycosylation in the presence of p-nitrophenyl  $\alpha$ -L-arabinofuranoside and various alcohols. Moreover, Rémond *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 (Rémond *et al.*, 2002; Rémond *et al.*, 2004). Moreover,  $\alpha$ -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  $\alpha$ -L-AFases in several biotechnological applications, it is perceived that research into exploring many aspects of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -L-AFases considered neither the economic nor the environmental aspects in the production of these enzymes. Furthermore, studies on bacterial  $\alpha$ -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  $\alpha$ -L-AFase production by bacteria grown under SSF and SmF are not available. Most of the reported  $\alpha$ -L-AFases are less stable at elevated temperature and has a narrow range of pH stability.

Nevertheless, a few of the reported  $\alpha$ -L-AFases were thermostable at high temperature. However, their substrate specificities were limited to one or two substrates. In fact, in addition to  $\alpha$ -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  $\alpha$ -L-AFase may provide potent  $\alpha$ -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  $\alpha$ -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.

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*Chapter 2*

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## Isolation, Screening and Characterization of Marine Bacteria that Produce $\alpha$ -L-Arabinofuranosidase

### 2.1 INTRODUCTION

The functional activity of bacteria can be regarded as one of the main factors that play key role in the cycling of organic matter and in nutrient regeneration in marine coastal and estuarine ecosystems. Moreover, a special feature of tropical estuaries such as the Mandovi estuary is the presence of evergreen trees of mangroves. The decomposition of continuously shed foliage from these trees by various microorganisms forming detrital matter makes this ecosystem rich in nutrients (Odum and Heald, 1975; Matondkar *et al.*, 1980). The bacteria present in such ecosystems utilize plants detrital matter breaking these into simpler mono, di, oligo and polysaccharides (Rawte *et al.*, 2002). Besides the naturally occurring tidal and salinity variation, the intense human activities, account for the physiologically diverse bacteria in these ecosystems (Rawte *et al.*, 2002). Most importantly, these bacteria produce a battery of enzymes that hydrolyze a variety of polymers in the detrital matter. Thus, the tropical marine coastal and estuarian bacteria could be expected to be diverse with respect to their enzymatic activities.

The hemicellulose and pectin polysaccharides are among various natural polymers in the detrital matter in marine coastal and estuaries ecosystems. These two polymers contain arabinose residues either as main and/or side chain components (Kroon and Williamson, 1996; Bonnin *et al.*, 2002; De Vries and Visser, 2001; Saha, 2003; Numan and Bhosle, 2006). The presence of arabinose side chains prevents the action of other hemicellulose and pectin degrading

enzymes (Kaji, 1984; Saha and Bothast, 1998a;b; Saha, 2000; Rahman *et al.*, 2003; Numan and Bhosle, 2006). The removal of arabinose side chains can be accomplished by the action of  $\alpha$ -L-arabinofuranosidases ( $\alpha$ -L-AFases) which facilitate the complete degradation of these polymers (Kaji, 1984; Beldman *et al.*, 1997; Saha, 2000). Hence, production of  $\alpha$ -L-AFase by bacteria is important for efficient utilization of hemicelluloses and pectins in marine coastal and estuaries ecosystems. Previous study indicated the potential of bacteria from these ecosystems for the production of xylanases (Khandeparkar and Bhosle, 2006a;b). However, little is known on the production of  $\alpha$ -L-AFase from bacteria isolated from these ecosystems.

The strategy of isolation of bacteria with potential to produce required enzyme usually involves use of the enrichment technique. The technique is based on the selection of suitable carbon source, specific growth conditions, and use of a sample of mud, soil or water (suppose to contain required bacteria) from a target environment. Once the growth is recorded, this is used as an inoculum to inoculate the fresh medium. This is repeated several times. The ability of bacteria to produce the required enzyme is then detected by suitable screening method. The present chapter deals with the isolation, screening and characterization of  $\alpha$ -L-AFase producing bacteria from marine sediments collected from the Dona Paula Bay and from a site called the four pillars along the Mandovi estuary, Goa.

## **2.2 MATERIALS and METHODS**

### **2.2.1 Preparation of agricultural substrates**

Oat bran, wheat bran and rice husks were obtained from flour-mill while sugar cane bagasse was obtained from farmhouse in Goa. The substrates were prepared as describe by Khandeparkar and Bhosle (2006a). The substrates were washed two to three times in distilled water and then boiled in distilled water for 10 to 15 min. The water was then decanted and substrates were dried in an oven 50°C and grinded using home mixer. Sugar beet pulp was prepared according to Buchholt *et al.* (2004). Beet roots purchased form local market was used to prepare sugar beet pulp. They were washed, sliced, hand-cut into strips and minced in a chopper through an 8 mm perforated plate. The minced material was immediately blanched at 85°C for 5-10 min to inactivate enzymes and denature plant protein. Free sugar was removed from the material by suspension in water at 60°C for 2 h. The pulp was then separated on a screen; washed; oven dried at 70°C and grinded using home mixer. All the grinded substrates were then sieved using a 40 µ mesh and stored at -20°C until used.

### **2.2.2 Enrichment of $\alpha$ -L-AFase producing bacteria**

Enrichment was carried out in Erlenmeyer flasks containing modified basal salt medium (MBSM) and 0.5% w/v of carbon source. The composition of MBSM is presented in **Table 2.1**. The carbon sources used included either of oat spelt xylan, arabinan, wheat bran, oat bran and sugar beet pulp. The medium was adjusted to pH 9.0 using 1 N NaOH and autoclaved at 121 °C for 15 min. After cooling, the medium was inoculated with of 1% w/v of sediment samples collected from the Mandovi estuary or the Dona Paula bay. The inoculated

medium flask was incubated on an orbital shaker (100 RPM) either at 50°C or at room temperature ( $28 \pm 2^\circ\text{C}$ ) over a period of 4 days. After growth was observed as visible turbidity, the medium was further sub-cultured thrice every 48 h into fresh MBSM containing the same carbon source to confirm consistency of bacterial growth.

**Table 2.1:** The chemical composition of **(a)** Modified Basal Salt Medium (MBSM), **(b)** Trace metals solution.

<b>(a) Modified Basal Salt Medium</b>	
<b>Chemical composition</b>	<b>per liter</b>
NaCl	25.00 g
KCl	0.75 g
MgSO <sub>4</sub>	7.00 g
NH <sub>4</sub> Cl	0.50 g
K <sub>2</sub> HPO <sub>4</sub> (10%)	7.00 ml
KH <sub>2</sub> PO <sub>4</sub> (10%)	3.00 ml
Yeast extract	0.60 g
Peptone	0.20 g
Trace metals solution*	1.00 ml
Distilled water	1000 ml
<b>(b) *Trace metals solution</b>	
<b>Chemical composition</b>	<b>per liter</b>
H <sub>3</sub> BO <sub>3</sub>	2.85 g
MnCl <sub>2</sub> .7H <sub>2</sub> O	1.80 g
FeSO <sub>4</sub> .7H <sub>2</sub> O	2.49 g
Na-Tartarate	1.77 g
CuCl <sub>2</sub>	0.03 g
ZnCl <sub>2</sub>	0.02 g
CoCl <sub>2</sub>	0.04 g
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.02 g

(Modified from Bhosle, 1981)

### **2.2.3 Isolation and purification of bacteria**

After obtaining consistent microbial growth, 1ml of the culture broth was appropriately diluted and spread plated on Zobell marine agar (ZMA) (Himedia, Mumbai, India) plates. These plates were incubated for 24 h at room temperature ( $28 \pm 2^\circ\text{C}$ ). The individual colonies were collected, re-streaked on ZMA to check their purity. The purity of the bacterial isolates was further confirmed by repeated plating on ZMA and microscopic examination after Gram staining. The pure cultures obtained were maintained on ZMA slants at  $4^\circ\text{C}$ . The bacterial isolates were divided into five groups depending on the carbon source used for their isolation and were named as wheat bran group (W), oat bran group (O), sugar beet pulp group (SBP), arabinan group (Ara) and oat spelt xylan group (OSX).

### **2.2.4 Screening for $\alpha$ -L-AFase producing bacteria**

In order to screen individual bacterial isolate for their ability to produce  $\alpha$ -L-AFase, each isolate was grown in MBSM and 0.5% w/v of either of wheat bran, oat bran, sugar beet pulp, arabinan and oat spelt xylan. The medium (20 ml) was added to several 100 ml Erlenmeyer flasks. The flask containing medium was sterilized at  $121^\circ\text{C}$  for 15 min. Flasks were inoculated with a loopfull of each isolate freshly pre-grown on ZMA plate. The inoculated flasks were incubated on orbital shaker (100 RPM) at either  $50^\circ\text{C}$  or room temperature ( $28 \pm 2^\circ\text{C}$ ) for 3 days. Each culture showing growth was then sub-cultured thrice using the same medium and growth conditions.

#### *Crude enzyme preparation:*

At the end of 3<sup>rd</sup> subculture, the growth medium was centrifuged at 10000 RPM at 4°C for 10 min. The supernatant was collected and used for enzyme estimation using the method described below.

#### **2.2.4.1 Estimation of $\alpha$ -L-AFase activity**

The  $\alpha$ -L-AFase activity in the supernatants was estimated following the method described by Gilead and Shoham (1995). The assay was based on the hydrolysis of *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (*p*-NPAF) (Sigma). The reaction mixture contained 80  $\mu$ l of appropriately diluted enzyme sample, 80  $\mu$ l of 50 mM sodium phosphate buffer (pH 8.0), and 40  $\mu$ l of *p*-NPAF (4 mg/ml) in the same buffer. Tubes were incubated at 80°C for 10 min, and the reaction was then terminated by the addition of ice-cold 1 M Na<sub>2</sub>CO<sub>3</sub>. The yellow color produced by the release of *p*-nitrophenol (*p*NP) was measured at 420 nm using UV-Vis spectrophotometer (Shimadzu, UV-1601).

**$\alpha$ -L-AFase unit:** One international unit (U) of  $\alpha$ -L-AFase activity was defined as the amount of enzyme which produces 1  $\mu$ mol of *p*-nitrophenol (*p*NP) per min under the standard assay conditions described above.

#### **2.2.5 Evaluation of bacterial isolates for $\alpha$ -L-AFase production under solid state fermentation**

The isolates were evaluated for their ability to produce  $\alpha$ -L-AFase when grown under solid state fermentation (SSF). For this purpose, carbon sources such as wheat bran, oat bran, sugar beet pulp, and oat spelt xylan were used. SSF was carried out in 100 ml Erlenmeyer flasks containing 1g of either of the above carbon source was moisturized with 3 ml of MBSM (substrate to moisture ratio

1: 3 w/v). The flasks were sterilized at 121°C for 15 min. Inoculum was prepared by growing the culture for 48 h in MBSM supplemented with 0.5% birch wood xylan, was used to inoculate SSF medium. The flasks were incubated under static condition at room temperature ( $28 \pm 2^\circ\text{C}$ ) for 72 h.

#### *Crude enzyme preparation*

At the end of the fermentation period a known weight of the fermented medium was suspended in a known volume of 50 mM phosphate buffer (pH 8) and vortexed for 5 min. The mixture was then centrifuged at 10000 RPM at 4°C for 10 min. The supernatant was collected and used for the estimation of  $\alpha$ -L-AFase following the standard assay described above.

### **2.2.6 Screening $\alpha$ -L-AFase bacterial producers for other xylanolytic enzymes**

The supernatant obtained as above was also used to evaluate the production of other xylanolytic enzymes such as xylanases and acetyl esterases. The xylanases and acetyl esterases were estimations following the methods described below.

#### **2.2.6.1 Xylanase activity estimation**

Xylanase activity was estimated following the procedure of Khandeparkar and Bhosle (2006a). The xylanase estimation was based on the hydrolysis of 1% birchwood xylan (Sigma). The reaction mixture contained 10  $\mu\text{l}$  of appropriately diluted enzyme sample and 250  $\mu\text{l}$  of 1% birchwood xylan dissolved in 50 mM glycine-NaOH buffer (pH 9.0). Tubes were incubated at 80°C for 10 min. The xylose released was estimated spectrophotometrically by using DNSA reagent (Miller, 1959).

**Xylanase unit:** One international unit (U) of xylanase activity was defined as the amount of enzyme which produces 1  $\mu\text{mol}$  xylose in 1 min under the standard assay conditions described above.

#### **2.2.6.2 Acetyl esterase activity estimation**

The acetyl esterase activity of the collected supernatants was estimated following the method described by Shao and Wegal (1995). The acetyl esterase assay was based on the liberation of 4-methylumbelliferone from 4-methylumbelliferyl acetate (Sigma). Assay mixtures containing 50 mM sodium phosphate buffer (pH 7.0) saturated with 4-methylumbelliferyl acetate (1 mmol in 1.3ml) and an enzyme preparation appropriately diluted in the same buffer (0.2ml). The reaction mixture was incubated at 70°C for 5 min after which 0.5 ml of ice-cold citric acid (50 mM) was added to terminate the reaction. The amount of fluorescence liberated was read at an excitation wavelength of 335 nm and an emission wavelength of 440 nm using fluorescence spectrophotometer (Hitachi, F-2000).

**Acetyl esterase unit:** One international unit (U) of acetyl esterase activity was defined as the amount of enzyme which liberates 1  $\mu\text{mol}$  of 4-methylumbelliferone per min under the assay conditions described above. Control samples were used during  $\alpha$ -L-AFase, xylanase and acetyl esterase enzymatic estimations. The reference samples used included substrate solution incubated without enzyme and diluted enzyme solution incubated without substrate in the buffer. The reading of the control sample was determined using the same procedure used for estimation of each of the enzyme. The value of the control sample was deduced from the value of the test sample. The activity of

$\alpha$ -L-AFase, xylanase and acetyl esterase was expressed as unit per gram of initially dried substrate (U/g IDS) when the culture was grown under SSF.

### **2.2.7 Characterization and identification of the bacterial isolates**

The isolated cultures that produce  $\alpha$ -L-AFase were examined microscopically for cell morphology after Gram staining (Harrigan and McCance, 1973). They were then studied for their morphological, biochemical and physiological characteristics using standard method (MacFaddin, 1980; Oliver, 1982). The identification of the isolates was done following *Bergey's manual of systematic Bacteriology* procedures (Sneath, 1994). Furthermore, gram negative bacterial isolates were tentatively identified using a taxonomic scheme for the identification of gram negative marine bacteria (Oliver, 1982). The spore-forming bacterial isolates were identified following *Bergey's Manual of Systematic Bacteriology* by using the methods described by Gordon *et al.* (1973), Claus and Berkeley (1986) and Gordon (1989). A scheme for identification of the aerobic spore-forming bacteria by phenotype published in *International Journal of Systematic and Evolutionary Microbiology* was further used for tentative identification of selected spore-forming bacilli (Reva *et al.*, 2001).

## **2.3 RESULTS and DISCUSSION**

### **Enrichment of $\alpha$ -L-AFase producing bacteria**

The tremendous range of catabolic diversity among microorganisms is one of the distinguishing features of the microbial world. The range of this diversity varies widely among individual species, from highly specialized ones that can utilize only one or a few substrates as energy sources to highly versatile species

that can utilize many compounds as the sole source of carbon and energy (Lynd *et al.*, 2002). Arabinose-containing substrates are widely available in different habitats. These habitats differ in their characteristics such as water availability, carbon sources, oxygen availability, redox potential, temperature variability, and nutrient status. Arabinose-containing substrates in turn differ in the type of arabinose linkages, in the amounts and distributions of arabinose side chains. These differences in habitats characteristics as well as in the structure of arabinose containing substrates may have fostered the development of different strategies for the utilization of these substrates. These strategies differ in enzyme architecture and presentation, rate and extent of arabinolysis, ancillary hydrolytic activities, fate of hydrolytic products, and interactions among arabinolytic and nonarabinolytic microbes (Uffen, 1997).

In this study, different arabinose containing substrates with emphasis on cheap agricultural substrates were used for the bacterial enrichment. The polymeric substrates such as arabinan and oat spelt were used as carbon sources for the isolation of bacteria with ability to produce  $\alpha$ -L-AFase. This is because of the presence of arabinose as the backbone and/or side chains in the polymeric structure of these polymeric substrates (Kaji, 1984; Saha, 2000; Numan and Bhosle, 2006). However, the agricultural substrates used for enrichment were also arabinose containing substrates. The polymeric substrates used are among the polymeric constituents of the agricultural substrates used for enrichment (Bonnin *et al.*, 1998; De Vries and Visser, 2001; Bonnin *et al.*, 2002; Saha, 2003). The agricultural substrates used included wheat bran, oat bran and sugar beet pulp. Wheat bran and oat bran are the substrates which are formed after industrial processing of wheat and oat grains. These grains are produced by

graminaceous plants and contain arabinoxylan (Saha, 2003). However, structural differences in arabinoxylans isolated from these substrates are reported previously (Timell, 1967; Izydorczyk and Biloderis, 1995; Bonnin *et al.*, 1998; De Vries and Visser, 2001; Saha, 2003; Adams *et al.*, 2004). The sugar beet pulp is an agro-industrial material which formed after industrial processing of beet root for sugar production. Sugar beet pulp usually contains high concentration of arabinose which is arranged in a polymeric structure called arabinan (Kroon and Williamson, 1996; De Vries and Visser, 2001; Bonnin *et al.*, 2002). The agricultural substrates were explored to attain some advantages. Common to arabinoxylans from wheat bran, oat bran and other plants sources, is the differences in the amounts, types, and distributions of arabinose side chains. These may result in biochemical diversity of  $\alpha$ -L-AFases that remove different arabinosyl residues from arabinose-containing substrates with different efficiencies (Kaji, 1984; Saha, 2000). Moreover, in nature, bacteria utilize arabinose present in arabinoxylan or arabinan which are usually associated with other polymeric structure forming hemicelluloses or pectin, respectively. The hemicelluloses and pectin are also associated with other polymeric structures including cellulose and lignin forming lignocelluloses. Thus, the  $\alpha$ -L-AFase produced by bacterial culture grown on these lignocelluloses may have good potential for industrial processing of lignocellulosic waste materials for production of useful products (Uffen, 1997). In addition, a variety of other hemicellulosic degrading enzymes are also expected to be produced by such bacterial cultures.  $\alpha$ -L-AFase produced by bacterial isolate with such enzymatic abilities was thought worthwhile to be investigated in this study. Previous studies indicated the potentials of utilization

of agricultural waste substrates for production of cellulases (Doppelbauer *et al.*, 1989; Krishna, 1999), xylanases (Archana and Satyanarayana, 1997; Gessesse and Mamo, 1999; Heck *et al.*, 2005; Khandeparkar and Bhosle, 2006a), amylase (Babu and Satyanarayana, 1995), pectinases (Solis-Pereira *et al.*, 1993; Kashyap *et al.*, 2003) and proteinases (George *et al.*, 1997; Sandhya *et al.*, 2005). However, isolation of bacteria that produce  $\alpha$ -L-AFase using these substrates was not explored previously. The induction of bacterial  $\alpha$ -L-AFases by these substrates was not explored adequately. Moreover, some of the agricultural substrates used in this study were formerly used to study  $\alpha$ -L-AFase induction in *Arthrobacter* sp in our laboratory (Kanderparker *et al.*, 2008). In contrast, scanty reports are available on production of  $\alpha$ -L-AFase by fungi grown on agricultural residues under SSF conditions (Roche *et al.*, 1994; Roche *et al.*, 1995; Filho *et al.*, 1996).

Using enrichment technique with these carbon sources, 73 bacterial isolates with the ability to produce  $\alpha$ -L-AFase, were isolated from the marine sediments collected from the Mandovi estuary and the Dona Paula Bay, west coast of India (**Table 2.2**). However, the  $\alpha$ -L-AFase producing isolates were accompanied with other bacterial isolates which could not produce  $\alpha$ -L-AFase. **Table 2.2** shows the marine bacterial isolates with the ability to grow on arabinose-containing substrates and produce  $\alpha$ -L-AFase. Some bacterial isolates did not produce  $\alpha$ -L-AFase but showed growth on arabinose containing substrates. This ability could be due to the action of other enzymes such as cellulases, xylanases and pectinases. On the other hand, the hydrolysis products produced by  $\alpha$ -L-AFase producing bacteria in growth media may stimulate the growth of other bacteria that are not  $\alpha$ -L-AFase producing bacteria and *vice versa*.

other bacteria that are not  $\alpha$ -L-AFase producing bacteria and *vice versa*. Because this study was concern with isolation of  $\alpha$ -L-AFase producing bacteria, the bacterial isolates which do not produce  $\alpha$ -L-AFase were not studied further. The  $\alpha$ -L-AFase activity produced by the  $\alpha$ -L-AFase producing isolates differed widely depending on the sample location, carbon source and bacterial isolates. The more potent producers were found to be related to the bacterial genera *Bacillus* (**Table 2.2**). These were obtained from the media that contained agricultural waste substrates as a carbon sources. Similarly, *Bacillus* isolates obtained by using other polymeric carbon sources also showed high  $\alpha$ -L-AFase production compared to other bacteria isolated from the same carbon sources. In aquatic environments, the settling of plant detritus to the sediment layer establishes a localized zone of enrichment that may be anoxic as a result of microbial activity. However, tidal changes in the shoreline of the aquatic environments may cause a change in oxygen availability in the localized zone of enrichment (Lynd *et al.*, 2002). These may favor the proliferation of facultative anaerobic arabinolytic bacteria such as *Bacillus* that withstand these changes. Thus, arabinolytic enzymes are presented more efficiently in these bacteria to maximize biosynthetic economy and the capture of hydrolytic products. The  $\alpha$ -L-AFase producing bacteria isolated from the Mandovi estuary were more efficient in  $\alpha$ -L-AFase production compared to those isolated from the Dona Paula Bay (**Table 2.2**). This was probably due to bacterial adaptation to the surrounding environment. The bacteria cultures isolated from the Mandovi estuary live in continued presence of detrital material derived from the mangrove plants. Other factor involved in this phenomenon is represented by human activities near the sampling site. The site was used to saw wood used for

construction activities. Therefore, organic matter derived from the mangroves and the dumped wood logs is the major source of arabinose containing lignocelluloses. The broad distribution of arabinolytic capability in this environment could suggest a conservation of an acquired capability in releasing arabinose from its substrates. More likely this is because of a convergent evolution toward an arabinolytic capability under the selective pressure of the abundant availability of arabinose-containing substrates in this environment (Uffen, 1997). These may reflect the diversity and potent ability of  $\alpha$ -L-AFase production by bacteria isolated from the Mandovi estuary.

#### **Characterization and identification of the bacterial isolates**

**Table 2.2** shows the morphological and physiological characteristics of  $\alpha$ -L-AFase producing bacteria. Genus assignment of the isolates was based on characteristics such as Gram reaction, colony and cell morphology, presence or absence of endospores, growth at high temperature, resistance to selected antibiotics, types of metabolism (oxidative vs fermentative), growth in the presence and/or absence of oxygen, presence or absence of enzymatic activities such as catalase, oxidase, and nitrate reductase, utilization of protein source such as trypton, utilization of carbon sources such as monosaccharide, disaccharides and polysaccharides, and other physiological and biochemical tests. The relative abundance of  $\alpha$ -L-AFase producing bacteria from the Dona Paula Bay and the Mandovi estuary sediments that utilize arabinose-containing substrates are shown in **Table 2.3**. Gram positive bacteria were dominant in the sediment samples when inoculated into any media containing agricultural substrates. Moreover, sporulated bacteria related to the genus *Bacillus* were

**Table 2.2: Morphological, physiological, biochemical characteristics and  $\alpha$ -L-AFase activity of isolated marine bacteria that utilize arabinose-containing polymers**

i.No	Isolate	Gram stain & Morphology	Motility	Spores	Oxidase	Catalase	Indole	MR	VP	Simmons Citrate	NO <sub>3</sub> Red.	Huge/Lifson	Starch	Gelatine	Pigment	Tentatively identified Genus	$\alpha$ -L-AFase (U/ml)
1	O-1	+ve rods	+	+	+	+	-	+	+	+	+	F	+	+	-	<i>Bacillus</i>	1.64
2	O-2	+ve rods	+	+	-	+	-	-	+	+	-	F	-	+	-	<i>Bacillus</i>	2.02
3	O-3	+ve rods	+	+	+	+	-	+	+	+	+	F	-	-	-	<i>Bacillus</i>	1.61
4	O-4	+ve rods	-	+	+	+	+	+	-	-	+	F	-	+	-	<i>Bacillus</i>	1.37
5	O-5	-ve rods	+	-	+	+	-	-	-	+	-	F	-	-	yellow	<i>Aeromonas</i>	0.012
6	O-6	-ve rods	-	-	+	+	-	-	+	-	+	-	-	-	-	<i>Alcaligenes</i>	0.018
7	O-7	-ve rods	+	-	+	+	-	-	-	+	-	O	-	-	-	<i>Pseudomonas</i>	0.032
8	O-8	+ve cocci	-	-	+	-	-	+	-	-	+	F	-	+	yellow	<i>Staphylococcus</i>	0.021
9	O-9	+ve coccobacilli	+	-	+	+	+	-	-	-	+	F	+	-	brown	<i>Unknown</i>	1.96
10	O-10	+ve rods	-	+	+	+	-	-	-	+	-	F	+	+	-	<i>Bacillus</i>	0.345
11	O-11	+ve rods	-	+	+	+	+	+	-	-	-	F	+	-	-	<i>Bacillus</i>	0.420
12	O-12	+ve rods	-	+	+	+	-	+	-	+	+	F	+	+	-	<i>Bacillus</i>	0.345
13	O-13	+ve cocci	-	-	+	+	-	-	-	+	-	O	+	+	yellow	<i>Staphylococcus</i>	0.048
14	O14	-ve rods	-	-	-	+	-	+	-	-	+	-	-	+	-	<i>Alcaligenes</i>	0.038
15	O-15	+ve rods	-	+	+	-	+	-	-	-	+	F	-	-	-	<i>Bacillus</i>	0.364
16	W-16	+ve rods	-	+	+	+	-	-	+	-	+	-	+	-	-	<i>Bacillus</i>	0.336

Table 2.2: Cont.

17	W-17	+ve rods	+	+	-	+	-	-	+	+	-	F	-	-	-	<i>Bacillus</i>	1.848
18	W-18	+ve rods	+	+	+	+	+	-	-	-	+	F	-	-	-	<i>Bacillus</i>	0.878
19	W-19	+ve rods	+	+	+	+	-	-	+	+	-	F	-	-	-	<i>Paenibacillus</i>	3.950
20	W-20	-ve rods	+	-	+	+	-	-	-	-	+	F	+	+	-	<i>Aeromonase</i>	0.014
21	W-21	-ve rods	+	-	+	+	-	-	+	+	-	O	-	+	-	<i>Pseudomonas</i>	0.024
22	W-22	+ve rods	-	+	+	+	-	+	-	+	-	F	-	+	-	<i>Bacillus</i>	0.747
23	W-23	-ve rods	+	-	+	+	-	-	+	-	-	O	+	-	-	<i>Pseudomonas</i>	0.008
24	W-24	+ve, rods	+	+	+	+	-	+	-	+	+	F	-	-	-	<i>Bacillus</i>	0.401
25	SBP-25	+ve, rods	-	+	+	+	-	+	-	-	-	F	+	-	-	<i>Bacillus</i>	0.654
26	SBP-26	-ve rods	-	-	+	+	+	-	-	+	+	F	+	-	-	<i>Aeromonase</i>	0.015
27	SBP-27	+ve, rods	-	+	+	+	+	-	+	+	+	F	+	+	-	<i>Bacillus</i>	0.682
28	SBP-28	-ve, rods	-	-	-	+	-	+	-	+	+	F	-	-	red	<i>Flavobacterium</i>	0.002
29	SBP-29	+ rod in chains	+	+	+	+	-	-	+	+	+	F	+	+	-	<i>Bacillus</i>	1.930
30	SBP-30	+ rod in chains	+	+	+	+	+	+	-	-	+	O	+	+	-	<i>Bacillus</i>	1.360
31	SBP-31	+ve rods	+	+	+	+	+	+	+	-	-	F	-	-	-	<i>Bacillus</i>	0.383
32	SBP-32	+ve, rods	-	+	+	+	+	-	-	+	+	-	-	-	-	<i>Bacillus</i>	1.050
33	SBP-33	+ve rod	+	+	+	+	-	-	+	+	-	F	-	+	-	<i>Bacillus</i>	3.110
34	SBP-34	+ve, rod	+	+	+	+	-	-	+	+	-	F	-	-	-	<i>Bacillus</i>	1.480
35	SBP-35	+ve, rod	+	+	+	+	-	-	+	+	-	F	+	-	-	<i>Bacillus</i>	3.410
36	SBP-36	+ve, rod	+	+	+	+	-	-	-	+	-	F	+	-	-	<i>Bacillus</i>	1.510
37	OSX-37	+ve rods	+	+	+	+	-	-	+	+	+NH	F	-	+	-	<i>Bacillus</i>	0.674
38	OSX-38	-ve, rods	-	-	+	+	-	-	-	-	-	F	+	+	-	<i>Arthrobacter</i>	0.153
39	OSX-39	+ve cocci	-	-	+	-	-	+	-	+	+	F	-	+	yellow	<i>Staphylococcus</i>	0.011

Table 2.2: Cont.

40	OSX-40	+ve cocci	-	-	+	-	+	-	-	+	+	F	-	-	yellow	<i>Staphylococcus</i>	0.022
41	OSX-41	+ve rods	-	-	-	-	+	+	-	-	+	F	-	-	-	<i>Bacillus</i>	0.114
42	OSX42	-ve rods	+	-	+	+	+	-	-	+	-	-	-	-	-	<i>Marinomonas</i>	0.011
43	OSX-43	+ve rods	+	-	+	+	-	+	+	+	+NH <sub>3</sub>	F	-	+	-	<i>Bacillus</i>	0.121
44	OSX-44	+ve cocci	-	-	+	-	-	-	+	-	-	F	-	+	yellow	<i>Staphylococcus</i>	0.011
45	OSX45	-ve, curved rod	+	-	+	+	-	+	-	-	-	-	-	-	-	<i>Marinomonas</i>	0.018
46	OSX-46	+ve rods	+	+	+	+	-	-	-	-	-	F	-	+	pink	<i>Bacillus</i>	0.467
47	Ara1	-ve rods	+	-	+	+	-	-	-	+	-	F	+	-	-	<i>Vibrio</i>	0.013
48	Ara 2	-ve coccobacilli	+	-	-	+	+	-	+	+	+	O	+	+	orange	<i>Chromobacterium</i>	0.007
49	Ara 3	-ve coccobacilli	+	-	+	+	+	-	-	+	+	O	+	+	-	<i>Chromobacterium</i>	0.004
50	Ara 6	-ve rods	+	-	+	+	+	-	-	+	+	F	+	+	-	<i>Aeromonas</i>	0.003
51	Ara 7	+ve rods	+	+	+	+	-	-	-	-	+	F	+	+	-	<i>Bacillus</i>	1.027
52	Ara 8	+ve, rods	+	+	+	+	-	-	-	-	+NH <sub>3</sub>	F	-	-	-	<i>Bacillus</i>	1.437
53	Ara 9	-ve coccobacilli	-	-	+	+	-	-	-	-	-	F	+	+	-	<i>Azotobacter</i>	0.018
54	Ara10	-ve rods	+	-	+	+	-	+	-	-	+	F	-	+	-	<i>Vibrio</i>	0.031
55	Ara 11	-ve coccobacilli	+	-	+	+w	+	-	-	+	+NH <sub>3</sub>	F	+	-	-	<i>Halobacter</i>	0.006
56	Ara12	-ve rods	+	-	+	+	+	+	-	-	-	F	+	+	-	<i>Vibrio</i>	0.011
57	Ara 13	-ve coccobacilli	-	-	+	+w	-	-	-	+	-	F	+	+	-	<i>Azotobacter</i>	0.023
58	Ara15	+ve rods	+	-	-	+	-	+	+	-	-	O	+	-	-	<i>Pseudomonas</i>	0.045
59	Ara 16	-ve Pleomorphic	+	-	+	+	-	+	+	+	+	F	+	-	-	<i>Arthrobacter</i>	0.004
60	Ara 17	-ve rods	-	-	+	+	-	-	-	-	-	F	+	+	yellow	<i>Photobacterium</i>	0.013

Table 2.2: Cont.

61	Ara18	-ve rods	-	-	+	+	-	-	+	+	-	O	+	+	-	<i>Pseudomonas</i>	0.029
62	Ara 20	+ve rods	-	-	+	+w	+	+	-	+	+	F	-	+	-	<i>Bacillus</i>	0.370
63	Ara 21	+ve rods	-	-	+	-	+	+	+	+	+	F	-	+	-	<i>Bacillus</i>	1.258
64	Ara22	-ve rods	-	-	+	+	+	-	-	+	-	O	-	+	-	<i>Pseudomonas</i>	0.015
65	Ara23	-ve rods	+	-	+	+	-	+	+	-	-	O	-	-	-	<i>Pseudomonas</i>	0.029
66	Ara24	-ve rods	+	-	+	+	-	-	+	-	-	O	+	-	-	<i>Pseudomonas</i>	0.016
67	Ara26	-ve rods	-	-	+	+	-	+	-	-	-	O	-	-	-	<i>Pseudomonas</i>	0.048
68	Ara 27	-ve rods	+	-	+	+	-	-	-	-	-	O	+	+	-	<i>Pseudomonas</i>	0.059
69	Ara 29	-ve rods	-	-	+	+w	-	-	-	-	+	F	-	-	-	<i>Chromohalobacter</i>	0.004
70	Ara31	-ve rods	+	-	+	+	-	-	-	+	-	O	+	+	-	<i>Pseudomonas</i>	0.025
71	Ara32	-ve rods	+	-	-	+	-	-	-	-	-	-	-	+	-	<i>Alcaligenes</i>	0.021
72	Ara 33	+ve rods	-	-	+	+m	+	-	+	+	+	F	+	+	-	<i>Bacillus</i>	1.051
73	Ara 35	-ve rods	+	-	+	+	-	-	+	-	+	F	+	+	-	<i>Vibrio</i>	0.012

l): oxidative; (F): fermentative; (+): positive reaction; (-): negative reaction; (w): weak reaction; (m): medium reaction

**Table 2.3:** Relative abundance of different isolated marine bacteria that utilize arabinose-containing substrates and produce  $\alpha$ -L-AFase

<b>Bacteria</b>	<b>T. No. of each isolate</b>	<b>% abundance among Gr +ve isolates</b>	<b>% abundance among Gr -ve isolates</b>	<b>% abundance among total isolates</b>
<i>Bacillus</i>	33	84.62	-	45.21
<i>Staphylococcus</i>	5	12.82	-	6.85
Unknown	1	2.56	-	1.37
<i>Pseudomonas</i>	11	-	32.35	15.07
<i>Vibrio</i>	4	-	11.76	5.48
<i>Chromobacterium</i>	2	-	5.88	2.74
<i>Chromohalobacter</i>	1	-	2.94	1.37
<i>Arthrobacter</i>	2	-	5.88	2.74
<i>Photobacterium</i>	1	-	2.94	1.37
<i>Flavobacterium</i>	1	-	2.94	1.37
<i>Azotobacter</i>	2	-	5.88	2.74
<i>Marinomonas</i>	2	-	5.88	2.74
<i>Alcaligenes</i>	3	-	8.82	4.11
<i>Halobacter</i>	1	-	2.94	1.37
<i>Aeromonas</i>	4	-	11.76	5.48
Total	73	100.00	100.00	100

most dominant in these samples. This may suggest that gram positive bacteria, especially the genus *Bacillus*, have more potential hydrolytic enzymes. These enzymes could be responsible for the ability of these bacteria to grow and utilize the agricultural substrates (De Vries and Visser, 2001; Saha, 2003). Furthermore, environmental conditions such as water availability and temperature variations could be among other factors that give *Bacillus* superiority to survive in such ecological niche. The dominant bacteria in such environment should have the ability to grow at extremes low or high levels of moisture and to be exposed to continuous changes in temperature for long periods. The physiological characteristics of *Bacillus* such as spore formation, their ability to adhere to and dominate the fermentation of insoluble

arabinoxylan particles are among other factors that make these bacteria the most dominant in such environment (Archana and Satyanarayana, 1997). High ratio (73.91%) of Gram positive bacteria that produce  $\alpha$ -L-AFase were isolated from media containing agricultural material and the polymeric substrate oat spelt xylan. In contrast, less Gram negative bacteria, (26.087%) producing  $\alpha$ -L-AFase belong to different genera were isolated from these media. Furthermore, the pure arabinan support the growth of both Gram positive and negative bacteria. However, Gram negative bacteria which produce  $\alpha$ -L-AFase were more dominant (85.19%) than the gram positive ones (14.815%) when arabinan was used as a carbon source for isolation of the cultures. **Table 2.3** also shows the relative abundance of each isolated genus among its Gram type of the isolated bacteria. The *Bacillus* was the most dominant among isolated Gram positive bacteria that produce  $\alpha$ -L-AFase. In contrast, the bacterial genera such as *Pseudomonas*, *Vibrio*, *Aeromonas* and *Alcaliegens* were the most dominant among isolated Gram negative bacteria that produce  $\alpha$ -L-AFase (**Table 2.3**). Most of these Gram negative isolates produce low level of  $\alpha$ -L-AFase especially when grown on agricultural substrates. However, slightly higher levels of  $\alpha$ -L-AFase were produced by some isolated Gram negative cultures such as *Pseudomonas* when grown on these substrates.

Utilization of solid state fermentation (SSF) for enzymes production offers many advantages over submerged fermentation (SmF) (Pandey, 1992; Pandey *et al.*, 1999; Pandey *et al.*, 2000; Pandey, 2001). Many of these advantages will be discussed in the coming chapters. Furthermore, so far only one report is available on  $\alpha$ -L-AFase production by bacteria grown on agricultural material under SSF. This is represented by a study conducted by our group on

$\alpha$ -L-AFase production from *Arthrobacter* sp grown under SSF (Kanderparker *et al.*, 2008). Considering, the advantages of SSF and the need for contribution in better understanding of bacterial  $\alpha$ -L-AFase production in SSF, the  $\alpha$ -L-AFase producing isolates were further tested for  $\alpha$ -L-AFase production under SSF condition. It was found that, only isolates belongs to *Bacillus* were able to grow and produce  $\alpha$ -L-AFase under these growth conditions (**Table 2.4**). Whereas, high levels of  $\alpha$ -L-AFase activities were obtained by *Bacillus* species, all other gram positive and gram negative isolates failed to grow under SSF condition. According to Archana and Satyanarayana (1997), this could be attributed to the ability of *Bacillus* to adhere to the substrate particles to produce filamentous cells for penetration, and to their specific need for water activity.

Some studies indicated the biotechnological importance of the synergistic action of arabinoxylan and arabinan degrading enzymes (Bachmann and McCarthy, 1991; Beldman *et al.*, 1997; Saha, 2000). Beside  $\alpha$ -L-AFase, xylanolytic enzymes such as xylanase and acetyl xylan esterase acts cooperatively for degradation of hemicelluloses (Saha, 2000; Numan and Bhosle 2006). Taking this into consideration, the bacterial isolates that grow under SSF and produced  $\alpha$ -L-AFase were further screened for their ability to produce xylanase and acetyl esterase enzymes. Production of  $\alpha$ -L-AFase, xylanase and acetyl esterase by these bacterial isolates is presented in **Table 2.4**. Nevertheless, all  $\alpha$ -L-AFase producing isolates that grow under SSF on agricultural substrates were able to produce xylanase. However, only a few of these isolates produced acetyl esterase when grown under the same conditions. The co-production of  $\alpha$ -L-AFase and xylanase by these isolates may suggest the presence of common inducer/s for both enzymes in the growth media. Previous studies indicated the

ability of pentose sugars arabinose and xylose and their polyols to induce the production of both enzymes under submerged fermentation (SmF) (Bachmann and McCarthy, 1991; Kulkarni *et al.*, 1999; Gueimonde *et al.*, 2007). As all the substrates used in SSF growth media contain both of these sugars, these might be responsible for the co-production of  $\alpha$ -L-AFase and xylanase by these isolates.

**Table 2.4:** Production of  $\alpha$ -L-AFase, xylanase and acetyl esterase by isolated marine bacteria with ability to grow under solid-state fermentation

Isolate	$\alpha$ -L-AFase U/g IDS	Xylanase U/g IDS	Acetyl esterase U/g IDS*	Isolate	$\alpha$ -L-AFase U/g IDS	Xylanase U/g IDS	Acetyl esterase U/g IDS
O1	6.08	2791	-	B33	11.29	3524	-
O2	8.17	1957	2937	B34	7.71	2279	-
O3	5.95	2435	-	B35	10.22	3811	-
O4	5.78	2860	-	B36	5.83	3357	1284
W17	9.14	3060	-	OSX37	7.59	2305	-
W19	14.68	4112	1789	OSX46	5.92	2509	-
B29	9.09	3194	2824	Ara8	3.24	1694	541
B30	6.85	1938	-				

**IDS\*:** Initially dried substrate.

A number of  $\alpha$ -L-AFase producing bacteria have been isolated from different environments. The gliding bacterium *Cytophaga xylanolytica* which produce  $\alpha$ -L-AFase was isolated from fresh water sediments (Renner and Breznak, 1998). The *Streptomyces* sp PC22 that produced  $\alpha$ -L-AFase was isolated from soil collected from a sugar cane field (Lauruengtana and Pinphanichakarn, 2006). The terrestrial bacteria that produce  $\alpha$ -L-AFase such as *Streptomyces diastaticus* (Tajana *et al.*, 1992) and *Bacillus subtilis* 3-6 (Kaneko *et al.*, 1994) were isolated from soil. The rumen colon and faces were the habitats for  $\alpha$ -L-AFase producing bacteria such as *Butyrivibrio fibrisolvens* (Dehority,

1966;1968), *Butyrivibrio fibrisolvens* GS113 (Hespell and O'Bryan, 1992), *Ruminococcus albus* 8 (Greve *et al.*, 1984) and *Prevotella ruminicola* B<sub>14</sub> (Gasparic *et al.*, 1995b). The probiotic *Bifidobacterium longum* that produce  $\alpha$ -L-AFase was isolated from human faeces (Gueimonde *et al.*, 2007). Nonetheless, not much is known about the habitats of other bacterial isolates which were reported to produce  $\alpha$ -L-AFase. This is because these bacteria were obtained from some culture collections and the habitats where they were isolated from were not mentioned in these reports. However, from the type of bacteria one may predict the bacterial habitat. For example, a thermophilic bacterium such as *Bacillus stearothermophilus* (Gilead and Shoham, 1995) and hyperthermophilic bacteria such as *Rhodothermus marinus* (Gomes *et al.*, 2000) and *Thermotoga maritime* (Miyazaki, 2005) are likely to be isolated from hydrothermal sources such as hot springs, volcano and hydrothermal vents.

The  $\alpha$ -L-AFase production by *Pseudomonas* was explored inadequately. To the best of our knowledge, only one report is available, so far on  $\alpha$ -L-AFase production by terrestrial *Pseudomonas cellulosa* (Beylot *et al.*, 2001a). A thermo-labile membrane bound intracellular  $\alpha$ -L-AFase was found to be produced by this bacterium. Hence, a study on more thermostable extracellular  $\alpha$ -L-AFase produced by marine *Pseudomonas* was thought to be worthwhile. Therefore, among the isolated gram negative bacteria, the marine bacterial isolate Ara27 that produces extracellular  $\alpha$ -L-AFase was selected for further studies. **Table 2.5** shows the morphological, physiological and biochemical characteristics of the isolate Ara27. This bacterium was found to be Gram negative, motile and slightly curved rods. It is obligatory aerobic, produce oxidase and catalase, hydrolyze starch and liquefy gelatin. The isolate can grow

in the presence of 5% of NaCl and can not grow at 41°C. These characteristics are similar to those described for *Pseudomonas* hence it was identified as *Pseudomonas* sp. The isolate Ara27 was deposited with the Marine Corrosion Material Research Department, National Institute of Oceanography, Goa, India which was designated as *Pseudomonas* sp NIOCC Ara27.

**Table 2.5:** Morphological, physiological, and biochemical characteristics of *Pseudomonas* sp NIOCC-Ara27.

Test	Results
Colony color	Off-white
Colony shape	Circular
Pigment	-
Cell shape	Rod
Gram stain	Gram -ve
Spore staining	-
Motility	+
Oxidase	+
Catalase	+
Huge-Leifson	Oxidative
Indole	-
Methyl red	-
Vogous Prauskaur	-
Simmons citrate	-
Nitrate reduction	-
Starch	+
Gelatin	+
Growth on 5% NaCl	+
Growth at 41°C	-
Fluorescence	-
Lipase (Tween 80)	-
Penicillin 10 U	Resistant
Streptomycin 10µg	Resistant
D-Glucose, D-Galactose, D-Fructose	+
D-Xylose, L-arabinose, D-arabinose	+
D-Maltose, D-Mannose, Sucrose	+
Cellobiose	+
L-Arabitol, D-Sorbitol	+
D-Mannitol, Inositol	-

Most of the reported bacterial  $\alpha$ -L-AFases were produced by cultures grown on expensive purified polymeric substrates such as arabinan, arabinogalactan, birch wood xylan and oat spelt xylan. In addition, submerged cultivation systems with low acidic to neutral pH were used in all of these studies. Moreover, most of these studies explored  $\alpha$ -L-AFase without taking into consideration other xylanolytic activities. Therefore, while selecting the best Gram positive strain for the production of  $\alpha$ -L-AFase several criteria were considered. These included level of extracellular  $\alpha$ -L-AFase activity at wide pH range, thermostability, level of extracellular xylanase and acetyl esterase and growth ability on a wide range of cheap agricultural substrates under SSF and SmF. Based on these criteria, isolate W19, was also selected for further studies. **Table 2.6** shows the morphological, physiological and biochemical characteristics of the isolate W19. This bacterium was found to be Gram positive rods, motile and produce swollen sporangia. The culture is facultative anaerobic with ability to grow in the presence of 5% NaCl and at temperature up to 50°C. The culture does not reduce nitrate to nitrite and does not hydrolyze starch or liquefy gelatin. It produces oxidase, catalase, and lipase, utilizes citrate and is positive for Vogous Prauskaur test. The culture has fermentative metabolism and produce acid from many sugars. These characteristics were found to be very similar to those described for *Bacillus* by Gordon *et al.* (1973) and Claus and Berkeley (1986) in Bergey's *Manual of Systematic Bacteriology* and Gordon (1989). Furthermore, a scheme for identification of the aerobic spore-forming bacteria by phenotype published in *International Journal of Systematic and Evolutionary Microbiology* was further used for identification of this bacterial strain (Reva *et al.*, 2001). According to this identification scheme,

the bacterial isolate W19 could be identified as *Paenibacillus azotofixans*. The bacterial isolate W19 is deposited with the Marine corrosion Material Research Department, National Institute of Oceanography, Goa, India which was designated as *Bacillus* sp NIOCCW19.

**Table 2.6:** Morphological, physiological, and biochemical characteristics of *Bacillus* sp NIOCCW19.

Test	Results
Colony color	Creamy
Colony shape	Irregular, undulate ,flat
Pigment	-
Cell shape	Rods
Gram stain	Gram +ve
Spores	+
Motility	+
Oxidase	+
Catalase	+
Huge -Leifson	Fermentative
Indole	-
Methyl Red	-
Vogous Prauskaur	+
Simmons citrate	+
Nitrate reduction	-
Starch hydrolysis	-
Gelatin liquefaction	-
Growth on 5% NaCl	+
Growth at 50 °C	+
Lipase	+
Sugar fermentation	
D-Glucose	A
D-Galactose	Alk
D-Fructose	A
D-Xylose	A
L-arabinose	A
D-arabinose	A
D-Maltose	A
D-Mannose	A
Sucrose	A
Cellobiose	A
D-Mannitol	A
D-Sorbitol	A
L-Arabitol	A
Inositol	-

(A : acid ; Alk: alkaline)

Based on the criteria described earlier these two cultures *Pseudomonas* sp NIOCCara27 and *Bacillus* sp NIOCCW19 isolated from the marine sediment samples from the Dona Paula Bay and the Mandovi estuary, respectively were selected for the studies described in the subsequent chapters.

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# *Chapter 3*

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## **Production, Isolation, Purification, Characterization and Substrate Specificity of $\alpha$ -L-Arabinofuranosidase from *Pseudomonas* sp NIOCCAr27**

### **3.1 INTRODUCTION**

Hemicelluloses, the second most abundant biomass on earth, represent 30-35% of lignocelluloses. Arabinan and arabinoxylan are the major polymers in hemicelluloses (Ward and Moo-Young, 1989; Saha, 2003). For efficient utilization of lignocelluloses, complete degradation of hemicellulose is essential. The major enzymes, degrading the backbone of arabinoxylan and arabinan are xylanases and endoarabinanses, respectively (Beldman *et al.*, 1997; Saha, 2000). The action of these enzymes is limited due to the presence of arabinose side chains present in these substrates (Saha and Bothast, 1998a;b; Saha, 2000; Rahiman *et al.*, 2003). The  $\alpha$ -L-arabinofuranosidases ( $\alpha$ -L-AFases) are glycoside hydrolases that remove arabinose residues from various pectic, homo-hemicellulosic polysaccharides (branched arabinans, debranched arabinans), heteropolysaccharides (arabinogalactans, arabinoxylans, arabinoxyloglucans, glucuronoarabinoxylans, etc.) and different glycoconjugates (Beldman *et al.*, 1997; Saha, 2000). The  $\alpha$ -L-AFases have a synergistic role assisting other lignocelluloses degrading enzymes in degradation of lignocelluloses. Therefore, they are considered as the promising tools in various agro-industrial processes (Aryon *et al.*, 1987; Saha, 2000). Furthermore, using  $\alpha$ -L-AFases 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). A variety of microorganisms was reported to produce  $\alpha$ -L-AFases that differ widely in their physiological role, biochemical properties and substrate specificity.  $\alpha$ -L-AFases have been isolated from many bacteria (Schyns *et al.*, 1994; Gilead and Shoham, 1995; Renner and Breznak, 1998; Degrassi *et al.*, 2003; Khandeparker *et al.*, 2008). However, not much is known about the production of  $\alpha$ -L-AFase by *Pseudomonas* spp. To the best of our knowledge, *Pseudomonas cellulosa* produces  $\alpha$ -L-AFase. Membrane bound  $\alpha$ -L-AFase produced by *P. cellulosa* showed wide substrate specificity (Beylot *et al.*, 2001a). However, the enzyme was very sensitive to temperature above 45°C and pH above 5.0. Furthermore, optimum temperature of the enzyme was very low (37°C). These findings limited the possibility of using this enzyme for industrial applications. Moreover, most of the enzymes used for industrial applications need to have the ability to work at elevated temperatures and pH (Haki and Rakshit, 2003). In view of this, there is a need for isolation of  $\alpha$ -L-AFases with novel properties from new microbial sources. In the previous chapter, potential of many marine bacteria to produce  $\alpha$ -L-AFases have been described. *Pseudomonas* sp NIOCCAr27 was one of the bacterial isolates that produced extracellular  $\alpha$ -L-AFase with properties different from those reported previously. The isolation, purification, characterization and substrate specificity of  $\alpha$ -L-AFase produced by this culture is presented in this chapter.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Culture growth and maintenance**

*Pseudomonas* sp NIOCCAr27 was routinely grown on MBSM supplemented with 0.5 % arabinan. The MBSM composition is given in **Table 2.1** of Chapter 2. The bacterium was maintained on Zoble Marine Agar (ZMA) (Himedia, Mumbai, India) slants at 4 °C.

### **3.2.2 Medium and growth conditions**

The cells of *Pseudomonas* sp were grown in the MBSM supplemented with 0.5 % arabinan (Megazyme) as the sole source of carbon and energy. The medium pH was adjusted to pH 7.0 using 1N NaOH and sterilized at 121°C for 15 min. A 0.5% (v/v) of the 24 h pre-grown cells of *Pseudomonas* sp in the same medium (2.7 O.D. at 540 nm) was used to inoculate a 250 ml flask containing 50 ml of the fresh growth medium. Flask containing inoculated medium was incubated on a rotary shaker 100 RPM, at room temperature ( $28 \pm 2^\circ\text{C}$ ) for 48 h.

### **3.2.3 Enzymatic assay**

$\alpha$ -L-AFase activity was measured as described in chapter 2. Enzyme estimation was conducted at 65°C and 50 mM Na- phosphate buffer (pH 7.0) was used for dissolving the chromogenic substrate *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (*p*-NPAF). One unit (U) of  $\alpha$ -L-AFase activity was defined as the amount of enzyme that liberated 1 $\mu$ mol of *p*NP at 65°C in 1 min.

### **3.2.4 Protein determination**

Protein was estimated by the bicinchoninic acid method with bovine serum albumin as a standard (Smith *et al.*, 1985).

### **3.2.5 Effect of carbon source on $\alpha$ -L-AFase production**

Effect of various carbon sources on the  $\alpha$ -L-AFase production was assessed by culturing the cells of *Pseudomonas* sp in MBSM supplemented with 0.5% of individual carbon source. The carbon sources used included arabinan (Megazyme), arabinogalactan (Fluca), oat spelt xylan, birch wood xylan, beech wood xylan (Sigma), wheat bran, oat bran, sugar beet pulp, rice husks, sugar cane bagasse, L-arabinose, D-arabinose, D-xylose, , cellulobiose, D-glucose, D-galactose, D-fructose, D-mannose, D-maltose, sucrose , lactose, L-arabitol, D-inositol, D-mannitol and D-sorbitol. Media pHs were adjusted to pH 7.0 using 1N NaOH prior to sterilization. A pre-grown culture in a medium with the same carbon source under investigation was used to inoculate each of the medium. Flasks were incubated on rotary shaker (100 RPM) at room temperature ( $28 \pm 2^\circ\text{C}$ ) for 84 h. At the end of 84 h growth, sample was collected, centrifuged at 10,000 RPM and  $4^\circ\text{C}$  for 10 min. The supernatant was used for the estimation of  $\alpha$ -L-AFase following the standard enzyme assay described earlier.

### **3.2.6 Optimization of growth medium for $\alpha$ -L-AFase production**

The concentrations of NaCl (0 to 40 mg/ml), inorganic nitrogen (0 to 2 mg/ml), inorganic phosphate (0 to 1.5 mg/ml), peptone (0 to 2 mg/ml), yeast extract (0 to 2 mg/ml) in MBSM and the medium pH were varied individually for the optimal production of the enzyme. This was conducted by varying the concentration of one nutrient while keeping the concentrations of other nutrients unchanged in the culture medium. Arabinan as a carbon source was used to conduct these

experiments. The medium (10 ml) in 50 ml flasks was inoculated with 0.5 % of pre-grown culture in the same medium. Flasks were incubated on rotary shaker (100 RPM) at room temperature ( $28 \pm 2^\circ\text{C}$ ) for 84 h. At the end of 84 h growth, sample was removed and centrifuged at 10,000 RPM and  $4^\circ\text{C}$  for 10 min. The supernatant was then used to estimate the  $\alpha$ -L-AFase activity following the standard method described earlier. The optimized medium obtained by these experiments is called hereafter Modified Basal Salt Medium No.1 (MBSM1). The composition of MBSM1 is given in Table 3.1.

**Table 3.1:** The composition of Modified Basal Salt Medium No.1 (MBSM1).

<b>Chemical composition</b>	<b>per liter</b>
NaCl	35.00 g
KCl	0.75 g
MgSO <sub>4</sub>	7.00 g
NH <sub>4</sub> Cl	0.50 g
K <sub>2</sub> HPO <sub>4</sub> (10%)	7.00 ml
KH <sub>2</sub> PO <sub>4</sub> (10%)	3.00 ml
Yeast extract	0.75 g
Peptone	0.50 g
Trace metals solution*	1.00 ml
Distilled water	1000 ml
pH adjusted with 1N NaOH	pH 7.0

\* Trace metal solution composition is given in Table 2.1b of Chapter 2.

### 3.2.7 Growth curve and $\alpha$ -L-AFase production

*Pseudomonas* sp was pre-cultured in the MBSM1 (Table 3.1) supplemented with 0.5 % arabinan for 48 h. The pre-cultured cells (1%) were used to inoculate 100 ml

of the same medium in 500 ml Erlenmeyer flasks. The flasks were incubated on orbital shaker (100 RPM) at room temperature ( $28 \pm 2^\circ\text{C}$ ) for 84 h. To monitor growth, culture samples were collected periodically and the absorbance was measured at 540 nm. Thereafter, the sample was centrifuged at 10,000 RPM for 10 min at  $4^\circ\text{C}$ . Supernatant was analyzed for  $\alpha$ -L-AFase activity following the standard assay described earlier.

To determine whether the enzymatic activity was predominantly secreted or cell-associated, the crude cell-extract was also prepared as described by Degrassi *et al.* (1995) and Degrassi *et al.* (2003). For that, the total  $\alpha$ -L-AFase activity was measured using the whole culture broth. Subsequently, cells were removed by centrifugation at 10000 RPM at  $4^\circ\text{C}$  for 10 min. The resulted supernatant was analyzed for  $\alpha$ -L-AFase activity. Cell associated  $\alpha$ -L-AFase activity was calculated as the difference between the total and cell-free supernatant.

### **3.2.8 Mass production and isolation of $\alpha$ -L-AFase from *Pseudomonas* sp**

*Pseudomonas* sp was grown in 5L Erlenmeyer flask containing 1L of MBSM1 supplemented with 0.5 % arabinan, pH 7.0. The flask was incubated on rotary shaker (100 RPM) at room temperature ( $28 \pm 2^\circ\text{C}$ ). The culture was harvested in mid stationary growth phase. After 72 h growth, the culture medium was centrifuged at 10,000 RPM for 10 min at  $4^\circ\text{C}$ . The supernatant was concentrated using Amicon ultrafiltration stirred cell (Amicon, USA) equipped with membrane filter (Millipore, Bedford, MA, USA) with 30 kDa MW cut-off. Stirred cell was pressurized at 50 psi using nitrogen gas. The enzyme was precipitated by adding ammonium sulfate to 80% saturation. The pellet was collected by centrifugation at

10,000 RPM at 4°C for 10 min. It was dissolved in 50 mM Na-phosphate buffer, pH 7.0, and dialyzed against the same buffer at 4°C for 24 h. Dialysis was conducted using standard cellulose-based dialysis tubing with a molecular weight cut-off of 13 kDa (Spectra/Pro). The dialysate was concentrated using rotary vacuum evaporator at room temperature ( $28 \pm 2^\circ\text{C}$ ).

### **3.2.9 Purification of $\alpha$ -L-AFase**

#### **3.2.9a Size exclusion or Gel filtration chromatography**

The concentrated dialysate was applied onto a column ( $65 \times 1.5$  cm) of Sephadex G-200 pre-equilibrated with 50 mM Na-phosphate buffer, pH 7.0. Loaded enzyme was eluted with same buffer, and the column was operated at a flow rate of  $1.5 \text{ ml min}^{-1}$ . Fractions (3 ml) were collected, and analyzed for  $\alpha$ -L-AFase. The protein content in fractions was estimated by measuring absorbance at 280 nm.  $\alpha$ -L-AFase containing fractions were pooled, dialyzed against 50 mM Na-phosphate buffer, pH 7.0, for 24 h, and concentrated using rotary vacuum evaporator. The concentrated enzyme sample was then applied onto an ion exchange column.

#### **3.2.9b Ion exchange chromatography**

The concentrated enzyme sample was loaded onto DEAE-Sephrose FF (Sigma-Aldrich Co.,USA) anion exchange column (15mm diameter  $\times$ 100mm high). The column was pre-equilibrated with 50 mM Na-phosphate buffer, pH 7, and operated at a flow rate of  $0.5 \text{ ml min}^{-1}$ . Elution was performed first with the 50 mM Na-phosphate buffer, pH 7, to remove unbound protein and thereafter with a linear NaCl gradient up to 1.5 M. Fractions (2ml) were collected and assayed for  $\alpha$ -L-AFase and protein content using the method described above. Active fractions

were pooled, dialyzed against 50 mM Na-phosphate buffer, pH 7.0, and concentrated using rotary vacuum evaporator to about (2ml). Concentrated enzyme was then applied onto CM –Sepharose FF (Sigma-Aldrich Co.,USA) cation exchange column (15mm diameter ×100mm high). The column was pre-equilibrated with 50 mM Na- phosphate buffer, pH 6.5. Column operation, enzyme elution, fractions processing and analysis procedures were as described for the DEAE –Sepharose FF chromatography. The purified enzyme was used for further studies described below.

### **3.2.10 Evaluation of purity and molecular mass of $\alpha$ -L-AFase**

SDS-PAGE electrophoresis was used to assess the purity of the enzyme. For molecular weight determination, SDS-PAGE (12%) electrophoresis was performed as described by Laemmli, (1970) using standard molecular weight markers (29 to 205 kDa) (Sigma -Cat.A7517). Proteins were visualized by staining with Coomassie brilliant blue (Sigma).

### **3.2.11 Native PAGE and zymogram analysis**

Native PAGE and zymogram analysis were performed using the modified method described by Bachmann and McCarthy (1991). Native polyacrylamide gels (7.5%) were constructed in the absence of SDS, and the volume was made up with distilled water. Samples were mixed with an equal volume of native sample buffer that did not contain SDS or  $\beta$ -mercaptoethanol. For gels that were to be developed as zymograms, samples were not boiled but were incubated at 50°C for 15-20 min in SDS sample buffer to permit dissociation without loss of enzyme activity. This was then loaded immediately onto the stacking gel and SDS was omitted from the

running buffer during electrophoresis. Immediately after electrophoresis, tracks containing boiled Molecular markers were removed and stained with Coomassie brilliant blue (Sigma). The remaining tracks were soaked in three 100-ml volumes of Na-phosphate buffer (50 mM, pH 7.0) at room temperature for 30 min. The polyacrylamide gel was sandwiched to the detection gel, which contained 1% agarose and 2.5 mM *p*-NPAF (Sigma) dissolved in Na-phosphate buffer (50 mM, pH 7.0).

### **3.2.12 Effect of temperature on $\alpha$ -L-AFase activity and stability**

The purified  $\alpha$ -L-AFase was used to assess the effect of temperature on the enzyme activity. The optimal temperature for the purified  $\alpha$ -L-AFase was obtained by assaying the enzyme activity at various temperatures between 30 and 75°C. Purified  $\alpha$ -L-AFase (0.4 U) and 2.5 mM of *p*-NPAF (80 $\mu$ L) were incubated at each selected temperatures and the activity was determined by the standard method described earlier. Prior to the enzyme activity determination, enzyme and its substrate were incubated separately in water bath at each respective temperature used for the assay. The thermal stability of the purified enzyme was assessed by incubating the enzyme at various temperatures between 40°C and 75°C for different time periods at pH 7.0. Enzyme aliquots were removed at definite time intervals and assayed for residual  $\alpha$ -L-AFase activity using standard method described earlier.

### **3.2.13 Effect of pH on $\alpha$ -L-AFase activity and stability**

The purified  $\alpha$ -L-AFase was used to assess the effect of pH on enzyme activity. The relative  $\alpha$ -L-AFase activity was assessed using 2.5 mM of *p*-NPAF at different

pHs. Three different buffers at concentration of 50 mM each were used. Citrate buffer was used for pH from 3 to 6; phosphate buffer was used for pH from 6 to 8, and glycine-NaOH buffer for pH 8 to 10. For determination of pH stability, the purified enzyme was diluted using respective buffer with pH range from 3 to 10 and incubated at room temperature ( $28 \pm 2^\circ\text{C}$ ). The residual activity of  $\alpha$ -L-AFase was assayed at particular time intervals during 12 h incubation period.

### **3.2.14 Kinetics studies**

Initial reaction rates of *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside hydrolysis were determined at different substrate concentration ranging from 0.08 mM to 4.0 mM of *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside in Na-phosphate buffer, (50mM, pH 7) at  $65^\circ\text{C}$ . The kinetic constants  $K_m$  and  $V_{\max}$  were estimated following the method of Lineweaver and Burk (1934).

### **3.2.15 Effect of Metal ions and other chemical agents**

The purified enzyme was incubated in 1 mM solution of either  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ , or EDTA for 1 h at room temperature ( $28 \pm 2^\circ\text{C}$ ). Residual activity was measured using standard enzyme assay described above.

### **3.2.16 Amino acid composition of $\alpha$ -L-AFase**

The amino acid composition of the purified  $\alpha$ -L-AFase was determined following the method of Khandeparkar and Bhosle (2006b). A known amount of the purified  $\alpha$ -L-AFase enzyme (100 $\mu$ l) was taken in the ampoule to which 2ml of 6N HCl was added. The ampoule was sealed after it was flushed with nitrogen gas. Hydrolysis

of the samples was carried out at 110 °C for 24 h. The hydrolyzate was then neutralized with 6N NaOH and centrifuged. The supernatant was evaporated to dryness under vacuum. The residue was dissolved in 2ml of HPLC grade distilled water, and filtered through a 0.4 µm polycarbonate filter (Nuclepore, USA). A suitable aliquot was then taken for the analysis of amino acids. Na-phosphate buffer (50 mM, pH 7) in place of enzyme was processed as above and used as blank. Prior to HPLC analysis, pre-column reaction of amino acids with *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol at pH 9.5 was carried out to form fluorescent derivatives. The Shimadzu HPLC model (LC-10A *VP*) consisting of a degasser, auto injector, column oven and fluorescent detector was used. A reverse-phase Shim-Pack HRC-ODS column (4.6mm i.d., 15 cm, 5 µm) with a ODS guard column (4.6mm i.d., 4.5 cm, 10µm) and a binary solvent system were used to separate amino acids. A binary solvent system consisting of 50 mM sodium acetate and 3% of tetrahydrofuran, pH 6.5 as solvent A and HPLC grade methanol as solvent B was used to separate amino acids. The best separation of amino acids was obtained with a flow rate of 1.5 ml/min using a 40 min gradient elution program beginning with 15% and ending with 100% solvent B, with a number of isocratic steps in the program. Amino acids were fluorometrically detected at an excitation wavelength of 328 nm and an emission wavelength of 450 nm. The identification of individual amino acids in the samples was determined by comparing the retention times of peaks in the samples with those of the standard solutions. Concentrations of the amino acids were calculated from peak areas relative to a known internal standard and a standard calibration using data handling system available with the

instrument. The derivatizing procedure used does not react with secondary amino acids like proline and hydroxyproline, so these were not quantified in the samples. The method showed a relative standard deviation of <10% for individual amino acids detected and quantified. All the glassware used was cleaned by soaking in 10% HCl for 24 h and rinsing with distilled water. Finally, all the glassware was rinsed with methanol and dried in a hot air oven.

### **3.2.17 Substrates specificity of $\alpha$ -L-AFase**

The action of  $\alpha$ -L-AFase on *p*-nitrophenyl aryl glycoside synthetic substrates,  $\alpha$ -L-arabinofuranooligosaccharides and arabinopolysaccharides was studied to determine the substrates specificity of the enzyme. Like *p*-NPAF other *p*-nitrophenyl aryl glycoside synthetic substrates were used to evaluate the action of  $\alpha$ -L-AFase on these substrates. The substrates including *p*NP- $\beta$ -D-xylopyranoside (*p*NP- $\beta$ -D-Xylp), *p*NP- $\alpha$ -L-arabinopyranoside (*p*NP- $\alpha$ -L-Arap), *p*NP- $\beta$ -L-arabinopyranoside (*p*NP- $\beta$ -L-Arap), *o*NP- $\beta$ -D-xylopyranoside (*o*NP- $\beta$ -D-Xylp), *p*NP- $\beta$ -D-glucopyranoside (*p*NP- $\beta$ -D-Glcp), *p*NP- $\alpha$ -D-galactopyranoside (*p*NP- $\alpha$ -D-Glcp), *p*NP- $\beta$ -D-galactopyranoside (*p*NP- $\beta$ -D-Galp), and the enzyme standard assay described earlier were used for this purpose.

#### **3.2.17a Evaluation of $\alpha$ -L-AFase action on $\alpha$ -L-arabinofuranooligosaccharides**

The action of the  $\alpha$ -L-AFase on  $\alpha$ -L-arabinofuranooligosaccharides was performed following the method described by Kaneko *et al.* (1998a,b).  $\alpha$ -L-arabinofuranooligosaccharides used for this purpose included  $\alpha$ -L-arabinofuranobiose,  $\alpha$ -L-arabinofuranotriose,  $\alpha$ -L-arabinofuranotetraose,

$\alpha$ -L-arabinofuranopentaose and  $\alpha$ -L-arabinofuranohexaose (All from Megazyme). Each reaction mixture contained 0.5 ml of a  $\alpha$ -L-AFase solution (0.4 U), 0.4 ml of Na-phosphate buffer (pH 7.0), and 0.1 ml of 1% any of the above substrates. After 12 h of incubation at 40°C, the reaction mixture was heated in a boiling water bath for 10 min to stop the reaction. Sample (1-2  $\mu$ l) of each mixture was used for thin-layer chromatography to characterize the hydrolysis products.

Ascending thin layer chromatography was performed using the silica gel plates (Kieselgel 60 F<sub>254</sub>, Merck, Darmstadt, Germany). The solvent system used was consisting of n-butanol–acetic acid– water (50:10:40, vol/vol/vol). The developed plate was sprayed with 5% sulphuric acid in methanol. Separated sugars on the plate were then detected by heating in oven at 120°C until the appearance of the spots.

### **3.2.17b Evaluation of $\alpha$ -L-AFase action on arabinofuranopolysaccharides**

The action of the  $\alpha$ -L-AFase on  $\alpha$ -L-arabinofuranopolysaccharides was studied following the methods described by Kaneko *et al.* (1998a;b) and Rahman *et al.* (2003).

#### *Preparation of the substrates:*

Soluble wheat arabinoxylan was prepared according to the manufacturer instructions. This is done by dissolving 100 mg of wheat arabinoxylan in 0.8 ml of 95% ethanol and 9 ml of deionized distilled water in a beaker. The beaker was loosely covered with aluminum foil. The mixture stirred continuously with magnetic stirrer on hot plate at 100°C for 10 min. After arabinoxylan was totally dissolved, the mixture was cooled to room temperature with continuous stirring.

The mixture volume was then adjusted to 10 ml to give final concentration of 1% wheat arabinoxylan. This is then stored in sealed container at 4°C.

Soluble oat spelt xylan was prepared according to the method of Yun-Sik *et al.* (2006). One gram of oat spelt xylan (Sigma) was added to Na-phosphate buffer (50mM, pH 7.0) so as to obtain 10% (w/v) oat spelt solution. The mixture was heated at 50°C for 60 min. After cooling to room temperature, the solution was centrifuged at 650×g at 22°C for 5 min to remove insoluble fraction. Soluble oat spelt xylan solution was then stored in sealed container at 4°C.

Either of sugar beet arabinan, sugar beet debranched arabinan (Megazyme), oat spelt xylan, birchwood xylan, beachwood xyaln (Sigma) or larchwood arabinogalactan (Fluka) was dissolved in Na-phosphate buffer (50mM, pH 7.0) so as to obtain 1 % (w/v) solution of each substrates. They were then stored in sealed containers at 4°C until used.

The action of  $\alpha$ -L-AFase on arabinose containing polysaccharides prepared as above was studied following the method described by Rahman *et al.* (2003). The reaction mixture contained 0.5ml of  $\alpha$ -L-AFase (0.4U), 0.4 ml of Na-phosphate buffer (50mM, pH 7.0) and 0.1 ml of 1% substrate. After 24 h of incubation at 40°C, the reaction mixture was boiled at 100°C for 10 min. Reaction mixture was then centrifuged at 10,000 RPM and used for determination of degradation products by TLC. TLC analysis of the reaction products was conducted as described earlier.

### **3.2.17c Hydrolysis rates of the enzyme acted on arabinose containing substrates:**

Hydrolysis rates of arabinofuranopolysaccharides were estimated as the rate of arabinose removal from each substrate by the action  $\alpha$ -L-AFase. This is done by

assuming the total content of arabinose in each substrate as 100% following Kaneko *et al.* (1998a;b). The glycosyl compositions of the substrates (according to manufacturers) were as follows: arabinan, Ara-Gal-Rha (81.3:13.1:3.1); debranched arabinan, Ara-Gal-Rha-GalUA (88:4:2:6); arabinoxylan, Ara-Xyl (41.0:59.0) and arabinogalactan, Ara-Gal-Rha (18.2:80.1:1.7). The reaction mixtures contained 0.5 ml of  $\alpha$ -L-AFase solution (0.4U), 0.4 ml of Na-phosphate buffer (50mM, pH 7.0), and 0.1 ml of 1% substrate (either of wheat arabinoxylan, arabinogalactan, arabinan, debranched arabinan, oat spelt xylan). Periodically, samples were withdrawn from each reaction mixture and analyzed for reducing sugar by Somogyi-Nelson method (1951).

### **3.3 RESULTS AND DISCUSSION**

#### **3.3.1 Effect of carbon source on $\alpha$ -L-AFase production**

*Pseudomonas* sp. was characterized by its ability to grow on most of the carbon sources used. Production of  $\alpha$ -L-AFase on different carbon sources is presented in **Table 3.2**. Agricultural substrates were effective in supporting bacterial growth. However, low level of enzyme activity was detected when these substrates were used as a single source of carbon. This was probably due to the complexity of these substrates that require more robust enzymatic system for efficient degradation of these substrates. Production of  $\alpha$ -L-AFase by *Pseudomonas* sp showed induction properties different from those reported from many other microorganisms. Arabinan and oat spelt xylan were the best inducers for  $\alpha$ -L-AFase production by *Pseudomonas* sp, while no significant induction was observed when L-arabitol,

**Table 3.2:** Effect of carbon sources on the production of  $\alpha$ -L-AFase by *Pseudomonas* sp.

<b>Carbon source</b>	<b>Activity (U/ ml)</b>
<b>Wheat bran</b>	<b>0.009</b>
<b>Oat bran</b>	<b>0.008</b>
<b>Sugar beet pulp</b>	<b>0.065</b>
<b>Sugar cane bagasse</b>	<b>0</b>
<b>Rice husks</b>	<b>0</b>
<b>Oat spelt xylan</b>	<b>0.031</b>
<b>Birchwood xylan</b>	<b>0.007</b>
<b>Beech wood xylan</b>	<b>0.009</b>
<b>Arabinan</b>	<b>0.143</b>
<b>Arabiongalactan</b>	<b>0</b>
<b>L-arabinose</b>	<b>0.010</b>
<b>D-arabinose</b>	<b>0.008</b>
<b>L-arabitol</b>	<b>0.006</b>
<b>D-xylose</b>	<b>0.010</b>
<b>D-sorbitol</b>	<b>0.002</b>

L-arabinose, L-sorbose, and wheat bran were used. These substrates induced  $\alpha$ -L-AFase activity in many microorganisms. For example, the best inducer for  $\alpha$ -L-AFase was L-arabitol for *Aspergillus terreus* (Luonteri *et al.*, 1995) and *Penicillium purpurogenum* (De Ioannes *et al.*, 2000); L-sorbose for *Trichoderma reesei* (Nogawa *et al.*, 1999); L-arabinose for *Bacillus stearothermophilus* (Gilead and Shoham, 1995) or wheat bran for *Arthrobacter* sp (Khandeparker *et al.*, 2008). L-Arabinose and L-arabitol are among common inducer for  $\alpha$ -L-AFase production in microorganisms (Gueimonde *et al.*, 2007). These compounds play a crucial role in the regulation of  $\alpha$ -L-AFase biosynthesis by bacteria and fungi (Saha, 2000). Among the substrates that were used in this study, only arabinan and oat spelt xylan were able to significantly induce the production of enzyme, while a very low

activity was detected in medium containing L-arabinose, L-arabitol, D-arabinose, D-xylose, D-sorbitol, wheat bran, oat bran, sugar beet pulp, Birchwood xylan and Beechwood xylan. Furthermore, all other substrate used could not induce  $\alpha$ -L-AFase production by *Pseudomonas* sp. Polysaccharides present in arabinan and oat spelt xylan induce the arabinose releasing enzymes in bacteria. Moreover, some of their degradation products induce the production of arabinose releasing enzymes. L-Arabinose is the final products of arabinan hydrolysis and a major product of oat spelt hydrolysis. This compound can be transported to the cytoplasm of bacterial cell to get reduced to L-arabitol. L-Arabitol is then further metabolized via L-xylulose, xylitol, D-xylulose and xylulose-5-phosphate respectively. The latter compound enters into the pentose phosphate pathway (Witteveen *et al.*, 1989; Raposo *et al.*, 2004; Gueimonde *et al.*, 2007). Moreover, all hexose monosaccharides and disaccharides inhibited  $\alpha$ -L-AFase production. This may indicates the production  $\alpha$ -L-AFase by *Pseudomonas* sp is inducible in nature. Furthermore, inhibition of  $\alpha$ -L-AFase production in the presence of hexose sugars was reported in some bacteria such as *Bacillus sterothermophilus* (Gilead and shoham, 1995); *Pseudomonas cellulosa* (Beylot *et al.*, 2001a) and *Bifidobacterium longum* (Gueimonde *et al.*, 2007).

### 3.3.2 Optimization of growth medium for $\alpha$ -L-AFase production

*Pseudomonas* sp. showed the ability to grow on some agricultural substrates and arabinopolysaccharides (Table 3.2). However, enzyme production was found to be higher when arabinan was used as a carbon source. Therefore, further studies were

conducted using this carbon source. Enzyme production by the bacterium was influenced by the nutrients in growth medium (Fig. 3.1a to Fig. 3.1e).

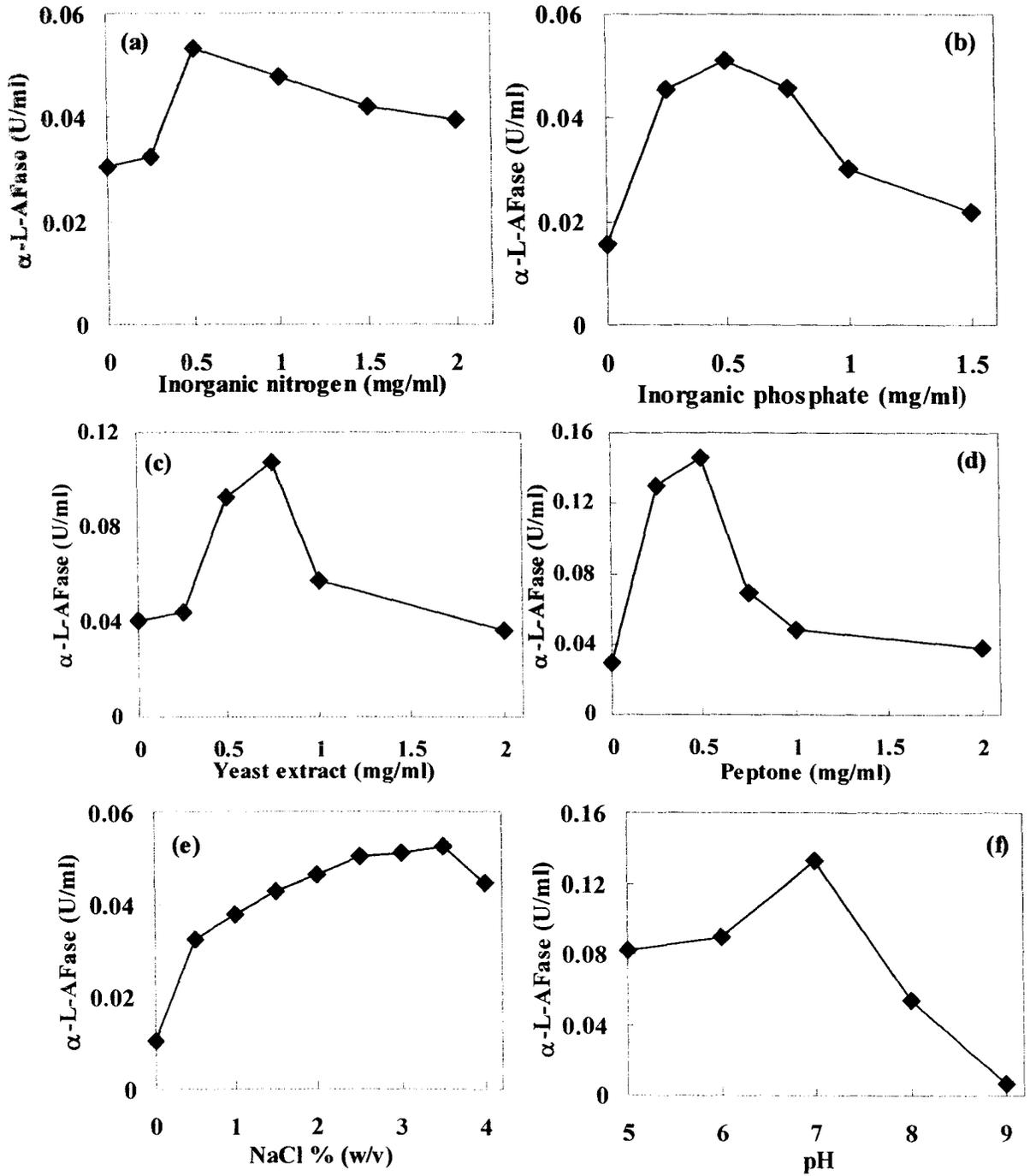


Fig.1: Effect of inorganic nitrogen (a), inorganic phosphate (b), yeast extract (c), peptone(d), NaCl (e) and medium pH (f) on  $\alpha$ -L-AFase production by *Pseudomonas* sp NIOCCAr27.

With increase in the concentrations of peptone; yeast extract and inorganic phosphates increased in  $\alpha$ -L-AFase production was observed. However, further increase in the concentration of these constituents, reduced the production of the enzyme (**Fig. 3.1a to Fig. 3.1d**). Moreover, high concentration of inorganic nitrogen moderately effected enzyme production. It was noticed that enzyme production increased with the increase in NaCl concentration and the maximal production was observed when 3.5% NaCl was present in growth medium (**Fig. 3.1e**). This could be due to the marine nature of this bacterium. In fact this is the concentration usually found in sea water. Moreover, initial pH of the growth medium highly influenced enzyme production. The bacterium showed maximal enzyme production when the medium was set at pH 7.0 (**Fig. 3.1f**). Higher pH values of the medium were found to strongly decrease enzyme production. This is may be attributed to the effect of pH on bacterial growth. Moreover, most Pseudomonads have an optimum pH for the growth of 5-7 (Beylot *et al.*, 2001a).

### **3.3.3 Growth curve and $\alpha$ -L-AFase production**

Growth of *Pseudomonas* sp and production of extracellular  $\alpha$ -L-AFase were studied using arabinan as a sole source of carbon. The culture showed a gradual increase in biomass, and  $\alpha$ -L-AFase production that reached the maximum at 48h.  $\alpha$ -L-AFase production remains more or less constant at this level up to 72 h of cultivation period. Although, a slight decrease in enzyme activity was noticed at the end of fermentation period. In contrast, bacterial biomass remains almost steady and showed no significant change over the cultivation period. There appears a good correspondence between  $\alpha$ -L-AFase production and the bacterial growth (**Fig. 3.2**).

Moreover,  $\alpha$ -L-AFase activity was quantified in the cell free supernatant, and whole culture broth. This is done to determine whether  $\alpha$ -L-AFase activity was predominantly extracellular or membrane bound. It was found that more than 95 % of the enzyme was secreted (Fig. 3.2). This indicates that,  $\alpha$ -L-AFase production by the bacterium was entirely extracellular. In contrast, *Pseudomonas cellulosa* (Beylot *et al.*, 2001a), *Thermomonospora fusca* (Tuncer, 2000; Tuncer and Ball, 2003a;b), and *Cytophaga xylanolytica* (Renner and Breznak, 1998) were found to produce membrane bound cell associated  $\alpha$ -L-AFases. These enzymes could be extracted from the cultures only by bacterial cells lysis. Furthermore, both extracellular and intracellular production of  $\alpha$ -L-AFase was reported from *Aureobasidium pullulans* (Saha and Bothast, 1998a).

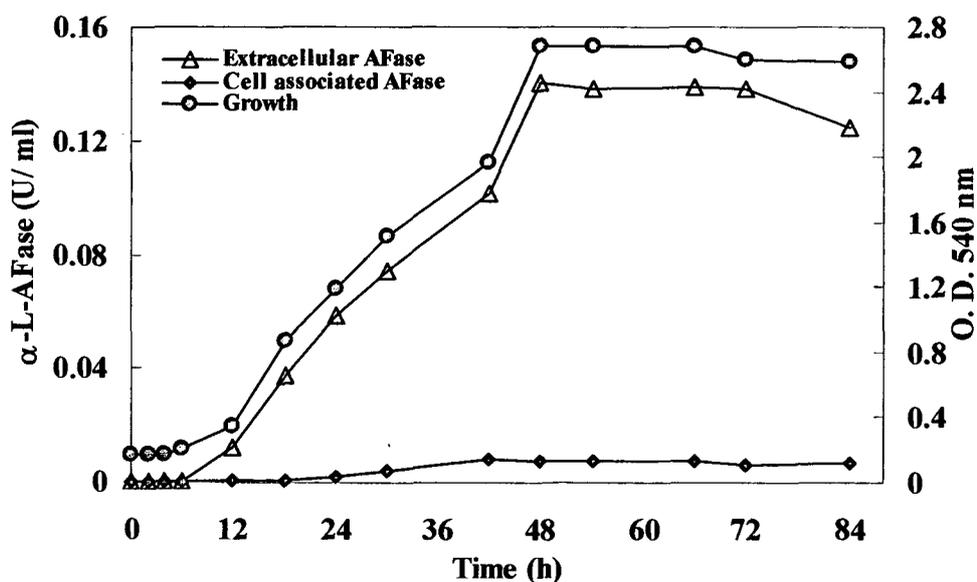


Fig. 3.2: Time course of growth and enzyme production by *Pseudomonas* sp.

### 3.3.3 Purification of $\alpha$ -L-AFase

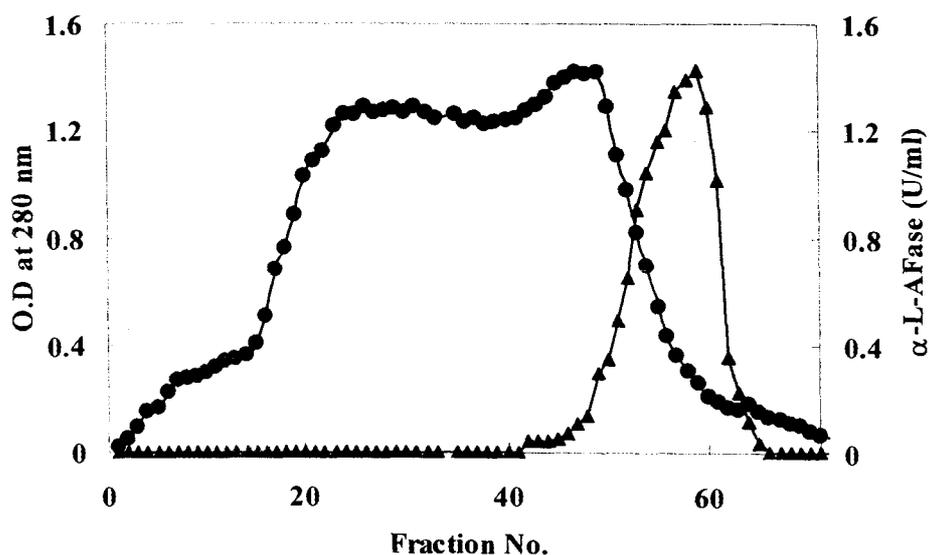
The isolation and purification of the enzyme were conducted at room temperature ( $28 \pm 2^\circ\text{C}$ ). The  $\alpha$ -L-AFase was purified to homogeneity from culture filtrate of

*Pseudomonas* sp grown on arabinan. A summary of the purification steps is presented in Table 3.3.

**Table 3.3:** Purification steps of  $\alpha$ -L-AFase enzyme isolated from *Pseudomonas* sp.

Steps	Enzyme activity U	Protein mg	Specific activity U/mg	Purification fold	Yield %
Supernatant	131.51	613.24	0.21	1.00	100.00
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	108.56	80.65	1.40	6.30	82.60
Sephadex-G200	87.44	31.14	2.80	13.10	66.50
DEAE Sepharose FF	72.63	5.08	14.30	66.70	55.20
CM Sepharose FF	27.73	1.05	26.40	123.10	21.10

The crude extract of the enzyme was concentrated by ultrafiltration using Amicon ultrafiltration stirred cell (Amicon, USA).  $\alpha$ -L-AFase activity was found only in the retentate. Ammonium sulfate precipitation of the enzyme increased the purification fold to 6.3. Gel filtration chromatography (Sephadex G-200) of the dialyzed precipitate resulted in one peak of  $\alpha$ -L-AFase activity (Fig.3.3a).



**Fig. 3.3a:** Elution profile of *Pseudomonas* sp  $\alpha$ -L-AFase on Sephadex G-200  
 (●) Protein profile at 280 nm (▲) Fractions containing  $\alpha$ -L-AFase activity

The protein peak was further purified on DEAE-Sepharose FF column chromatography (pH 7.0).  $\alpha$ -L-AFase activity which bound to the matrix of this column was eluted in the post gradient wash with NaCl (1.5M) (Fig. 3.3b). Further purification of  $\alpha$ -L-AFase was achieved by CM- column Sepharose FF (pH 6.5). During chromatography on this column;  $\alpha$ -L-AFase activity was eluted in the pre-gradient wash with Na-phosphate buffer (pH 6.5) as a single sharp peak (Fig. 3.3c). The overall level of the yield was 21.1 % while 123.1 purification fold of  $\alpha$ -L-AFase was achieved with specific activity of 26.4 U/mg. The purification results suggest that the enzyme preparation from *Pseudomonas* sp contains only one form of  $\alpha$ -L-AFase as no other active peak was detected during each purification step. Multiple forms of  $\alpha$ -L-AFase have been detected in the culture broth of *Aspergillus nidulans* (Ramon *et al.*, 1993), *Aspergillus niger* (Rombouts *et al.*, 1988), *Aspergillus terreus* (Luonteri *et al.*, 1995) and *Penicillium capsulatum* (Filho *et al.*, 1996).

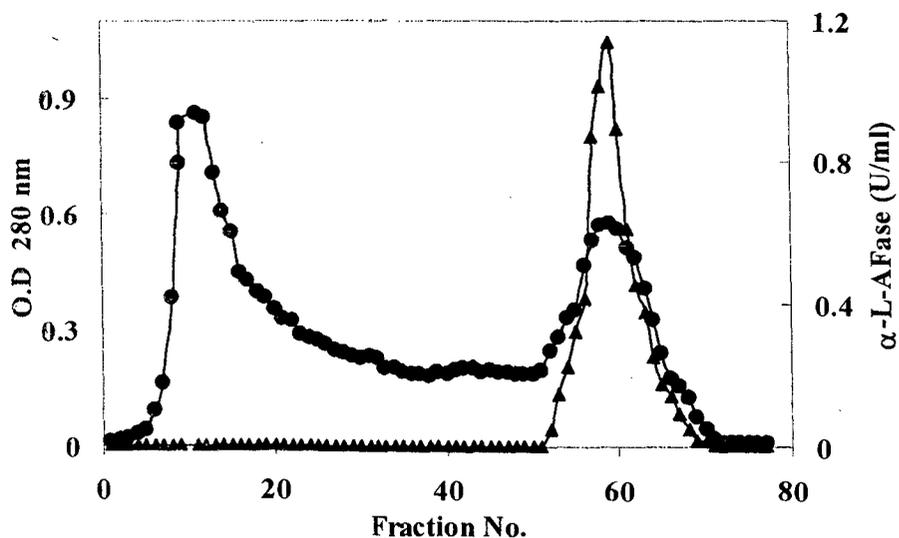
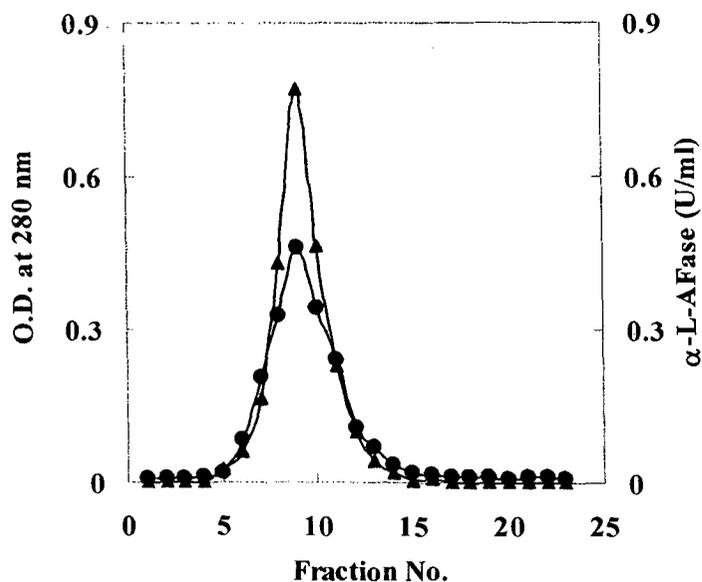


Fig. 3.3b: Elution profile of *Pseudomonas* sp.  $\alpha$ -L-AFase on DEAE-Sepharose FF. (●) Protein profile at 280 nm (▲) Fractions containing  $\alpha$ -L-AFase activity.

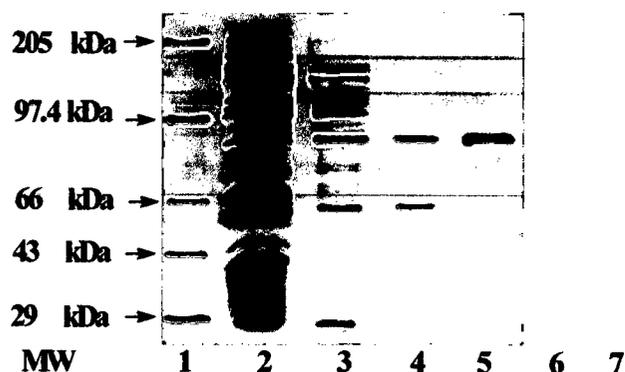


**Fig. 3.3c:** Elution profile of *Pseudomonas* sp  $\alpha$ -L-AFase on CM-Sepharose FF. (●) Protein profile at 280 nm (▲) Fractions containing  $\alpha$ -L-AFase activity.

### 3.3.4 Molecular weight determination and Zymogram analysis

The molecular weight of the purified enzyme was estimated from the relative mobility of the standard proteins on SDS-PAGE and native-PAGE. Following SDS-PAGE on 12 % polyacrylamide gels, enzyme preparation yielded a single band when stained for protein with Coomassie blue (**Plate 3.1**). The MW was  $\sim$ 84 kDa. Enzyme preparation also yielded a single band of protein coincident with a positive stain for activity when subjected to nondenaturing electrophoresis (**Plate 3.1**). As one single band of the enzyme was detected on both denaturated and native electrophoresis, it was suggested that the enzyme is a monomer. Monomeric  $\alpha$ -L-AFase was reported from few bacteria which include *Clostridium acetobutylicum* ATCC 824 with MW of 94 kDa (Lee and Forsberg, 1987) and *Arthrobacter* MTCC 5214 with MW of  $\sim$ 95kDa (Khandeparker *et al.*, 2008). However, several monomeric  $\alpha$ -L-AFase were isolated from fungi including *A.*

*A. niger* ( MW 67 kDa) (Kaneko *et al.*, 1993); *Monillinia frucigena* (MW 40 kDa) (Kelly *et al.*, 1987) and *P. purpurogenun* (58 kDa) (De loannes *et al.*, 2000).



**Plate 3.1:** SDS-PAGE and Zymogram analysis of purified  $\alpha$ -L-AFase from *Pseudomonas* sp. Lane1; Molecular markers; Lane2; after  $(\text{NH}_4)_2\text{SO}_4$  precipitate; Lane3; after sephadex G-200; Lane4; DEAE-Sepharose FF; Lane5; after CM-Sepharose FF; Lane6; Zymogram of the enzyme from crude extract; Lane6; Zymogram of the enzyme from the crude extract, Lane7; Zymogram of pure enzyme.

As compared to *Pseudomonas* sp, monomeric  $\alpha$ -L-AFase, several multimeric  $\alpha$ -L-AFases have been isolated from bacteria with molecular masses ranging from 110 kDa for  $\alpha$ -L-AFase from *B. stearotherophilus* T-6 (Gilead and Shoham, 1995) up to 495 kDa for  $\alpha$ -L- AFase from *S. purpurascens* IFO 3389 (Komae *et al.*, 1982). Bacterial multimeric  $\alpha$ -L-AFases were reported to posses either di, tetra, hexa, or octameric units. Dimeric  $\alpha$ -L-AFase was recorded from *B. stearotherophilus* L1 with MW of 110 kDa and consists of two subunits (MW 52.5 kDa and MW 57.5kDa) (Bezalel *et al.*, 1993).  $\alpha$ -L-AFase with tetrameric units was reported from *B. stearotherophilus* T-6 consists of four identical subunits (MW 64 kDa) (Gilead and Shoham, 1995). However,  $\alpha$ -L-AFase with hexameric

units were recorded from *Bacteroides xyloxyticus*X5-1 (Schyns *et al.*, 1994) and *Clostridium thermocellum* (Taylor *et al.*, 2006). Octameric  $\alpha$ -L-AFases with eight units were recorded from *Butyrivibrio fibrisolvens* GS113 with MW 240 kDa (consists of eight subunits, each with MW of 31 kDa) (Hespell and O'Bryan, 1992) and from *S. purpurascens* IFO 3389 with MW of 495 kDa (contains eight equal subunits of MW 65 kDa) (Komae *et al.*, 1982). *Pseudomonas* sp was found to produces only one form of  $\alpha$ -L-AFase as no other active band was detected on native PAGE of each purification step preparations (**Plate 3.1, Lane 6,7**). Multiple forms of  $\alpha$ -L-AFase have been detected in the culture broth of *S. diastaticus* (Tajana *et al.*, 1992); *A. awamori* (Kaneko *et al.*, 1998b); *A. nidulans* (Ramon *et al.*, 1993); *A. niger* (Rombouts *et al.*, 1988); *A. terreus* (Luonteri *et al.*, 1995); *P. capsulatum* (Filho *et al.*, 1996) and *Sclerotinia fructigena* (Laborda *et al.*, 1973).

### **3.3.5 Effect of temperature on the activity and stability of $\alpha$ -L-AFase**

The purified  $\alpha$ -L-AFase from *Pseudomonas* sp. showed very low activity at room temperature 30°C. The activity of the enzyme increased with the increase in temperature and the optimal activity was observed at 65°C. As the temperature increased over 65°C, the enzyme activity decreased, and reached 70% of the optimal activity at 75°C (**Fig. 3.4a**). The enzyme showed complete stability at 40°C for more than 12 h. The stability decreased when the temperatures was more than 55°C. The enzyme retained more than 90% of its activity at 55°C for more then one h, while only 45% enzyme activity was retained after incubation at 75°C for 20 min

(Fig. 3.4b). Beylot *et al.* (2001a) reported that  $\alpha$ -L-AFase from *P. cellulosa* had lower optimum temperature (37°C), and was rapidly deactivated at temperatures above 45°C.

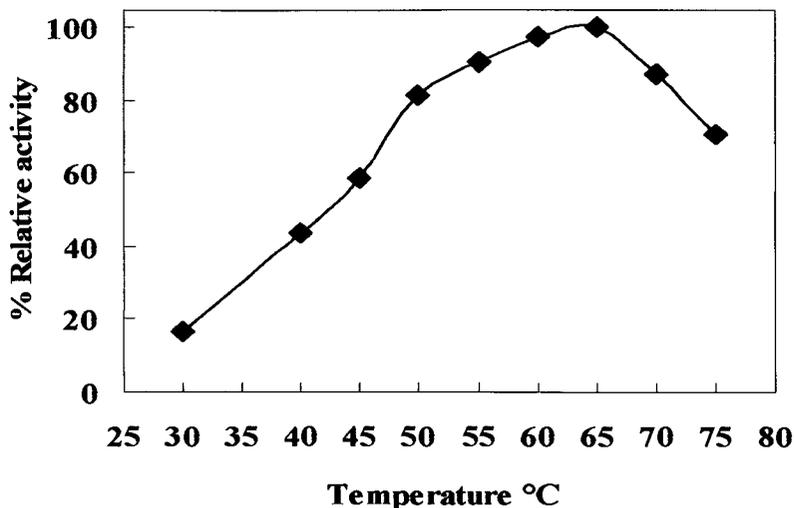


Fig. 3.4a: Effect of temperature on the activity of  $\alpha$ -L-AFase of *Pseudomonas* sp.

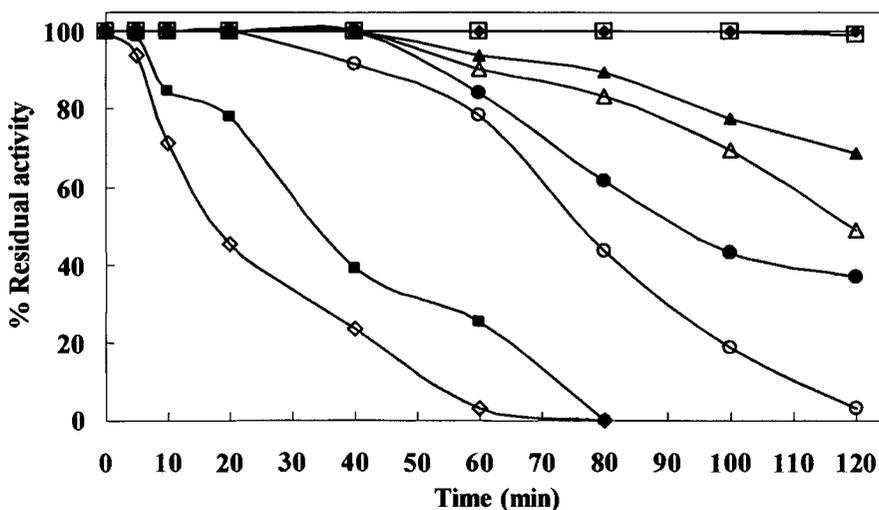


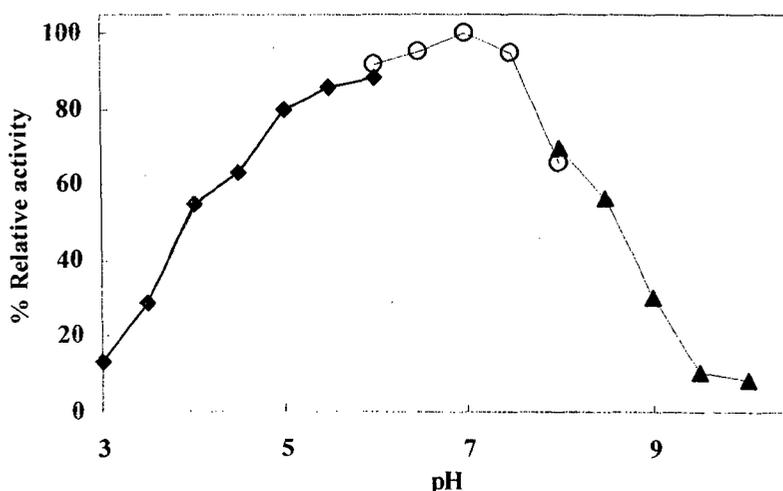
Fig. 3.4b: Thermal stability of  $\alpha$ -L-AFase from *Pseudomonas* sp.. The pure enzyme was incubated in 50mM Na-phosphate buffer, pH 7.0 at 40°C (◆) 45°C (■) 50°C (□) 55°C (▲) 60°C (△) 65°C (●) 70°C (○) 75°C (◇) for different intervals and residual activity was determined at 65°C and pH 7.0.

Moreover, higher optimal activities and temperature stability were reported from two thermophilic bacteria. This included  $\alpha$ -L-AFase from *Rhodothermus marinus* which had an optimum temperature of 85°C and had a half-life of 8.3 h at the same temperature (Gomes *et al.*, 2000). The  $\alpha$ -L-AFase isolated from *Thermotoga maritima* had a temperature optima at 90°C with 100 % stability at the same temperature over a period of 24h (Miyazaki, 2005). Moreover, the mesophilic bacterium *Arthrobacter* sp was reported to produce  $\alpha$ -L-AFase with optimal activity at 80°C and stability at 50°C over a period of 24 h (Khandeparker *et al.*, 2008). In fact, all other microbial  $\alpha$ -L-AFases have shown a broad range of temperature stability, with optimal activities occurring between 40–75°C (Kaji, 1984; Lee and Forsberg, 1987; Bezalel *et al.*, 1993; Fernandez-Espinar *et al.*, 1994; Filho *et al.*, 1996; Saha and Bothast, 1998b). Furthermore,  $\alpha$ -L-AFases from most fungi were reported to have optimal activities between (45-55°C). These included the  $\alpha$ -L-AFases from *A. oryzae* (Matsumura *et al.*, 2004), *A. Kawachii* (Koseki *et al.*, 2003), *Fusarium oxysporum* (Chacòn-Martènez *et al.*, 2004), *P. purpurogenum* (De Ioannes *et al.*, 2000), *P. chrysogenum* (Sakamoto and Kawasaki, 2003) and *Rhizomucor pumilus* HHT1 (Rahman *et al.*, 2003). In contrast, some  $\alpha$ -L-AFases from bacteria and actinomycetes were reported with higher temperature (55-75°C) optima. These included the  $\alpha$ -L-AFases from *B. pumilus* PS213 (Degrassi *et al.*, 2003), *Thermoanaerobactere ethanolicus* JW200 (Mai *et al.*, 2000), *Thermobacillus xylaniliticus* D3 (Debeche *et al.*, 2000), Bacterium PRI-1686 (Birgisson *et al.*, 2004), *S. chartreusis* GS901 (Matuso *et al.*, 2000), *Streptomyces thermaviolaces* OPC-520 (Tsujiibo *et al.*, 2002) and *T. fusca* (Tuncer and Ball,

2003a). Although a strict comparison of thermostability is not always possible due to the variations in the methodologies used by different authors; the optimum temperature and thermostability of *Pseudomonas sp.* NIOCCAr27  $\alpha$ -L-AFase appeared to be better than that of  $\alpha$ -L-AFase isolated previously from *P. cellulosa* (Beylot *et al.*, 2001a).

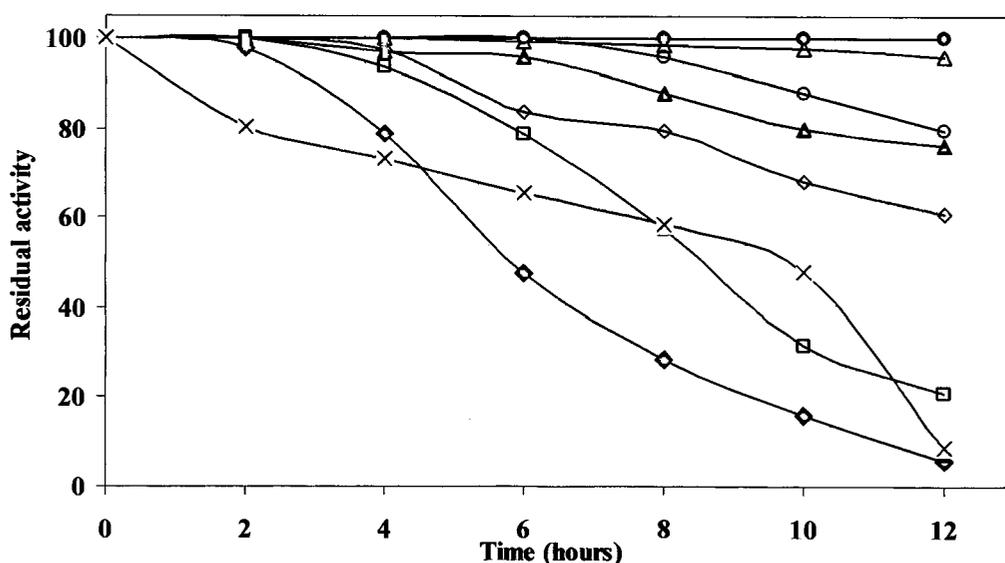
### 3.3.6 Effect of pH on the activity and stability of $\alpha$ -L-AFase

The  $\alpha$ -L-AFase from *Pseudomonas sp.* showed a pH optima of 7.0 (Fig. 3.5a). In contrast,  $\alpha$ -L-AFase from *P. cellulosa* had pH optima of 5.5. Moreover, other bacterial  $\alpha$ -L-AFases have a broad range of pH tolerance, with optimal activities occurring between pH 5.5 to 7.0. These included  $\alpha$ -L-AFases of the *Bacillus stearothermophilus* (6.5) (Bezalel *et al.*, 1993), *B. pumilus* PS213 (7.0) (Degrassi *et al.*, 2003), *Colsteridium cellulovorans* (6.0) (Kosugi *et al.*, 2002), and Bacterium PR1-1686 (6.0) (Birgisson *et al.*, 2004), *T. maritima* (7.0) (Miyazaki, 2005),



**Fig. 3.5a:** pH profile *Pseudomonas sp.*  $\alpha$ -L-AFase. Enzyme activity was measured at 65°C using different buffers. The buffers used were: ■, Citrate; ○, Phosphate; ▲, Glycine- NaOH.

*Thermobacillus xylaniliticus* D3 (6.2) (Debeche *et al.*, 2000) and *Bifidobacterium longum* B667 (6.0) (Margolles and de los Reyes-Gavilán, 2003). Furthermore,  $\alpha$ -L-AFase with higher pH optima (8.0) was reported from *Arthrobacter* sp. (Khandeparker *et al.*, 2008).  $\alpha$ -L-AFase from *Pseudomonas* sp found to be completely stable for more than 12 h at pH 7.0 at room temperature ( $28 \pm 2^\circ\text{C}$ ). It retained almost 100% of its activity at pHs from 5.0 to 9.0 for 4 h. The activity of the enzyme decreased sharply at extreme acidic or alkaline pHs which reached 47 % and 65% after 6 h incubation at pHs 3 and 10 respectively (Fig. 3.5b). Compare to pH stability profiles of  $\alpha$ -L-AFases reported from some bacteria, the  $\alpha$ -L-AFase from *Pseudomonas* sp has a wider range of pH stability.

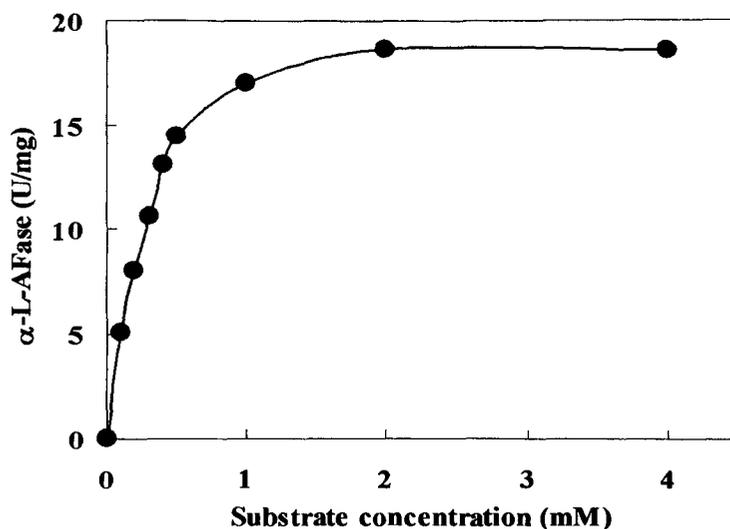


**Fig. 3.5b:** Effect of pH on stability of *Pseudomonas* sp.  $\alpha$ -L-AFase. The pure enzyme was incubated at room temperature up to 12 h in 50mM buffers. Buffers used were Citrate with pH 3 (♦), pH 4 (□) and pH 5 (Δ); Phosphate with pH 6 (△), pH 7 (○) and pH 8 (○); Glycine-NaOH with pH 9 (◇) and pH 10 (×). Residual activity was assayed at pH 7.0 and  $65^\circ\text{C}$ .

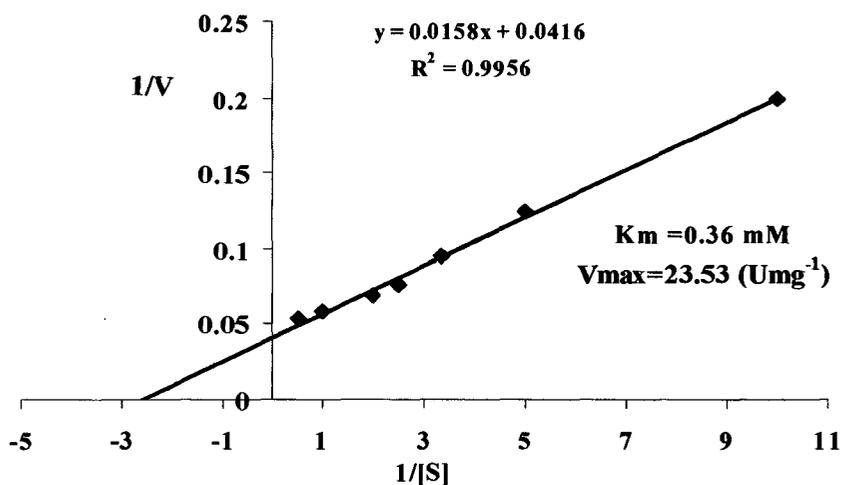
For example,  $\alpha$ -L-AFase from *B. pumilus* completely lost its activity at pH lower than 4.5 (Degrassi *et al.*, 2003). Furthermore, few  $\alpha$ -L-AFases were reported which showed similar pH stability profile. For example,  $\alpha$ -L-AFase from *R. marinus* was completely stable for 24 h at pHs between 5 and 9 (Gomes *et al.*, 2000).

### 3.3.7 Kinetic parameters

The rate dependence of the enzymatic reaction on the p-nitrophenyl- $\alpha$ -L-arabinofuranoside concentration at pH 7.0 and 65°C followed Michaelis-Menten kinetics. The  $V_{max}$  and  $K_m$  values were obtained from Lineweaver-Burke plots of the  $\alpha$ -L-AFase activity at 65°C using various concentration of p-nitrophenyl- $\alpha$ -L-arabinofuranoside.  $\alpha$ -L-AFase of *Pseudomonas* sp showed a  $V_{max}$  of 23.53 (U/mg) and  $K_m$  of 0.36 mM (Fig. 3.6a and Fig. 3.6b). The  $V_{max}$  of *Pseudomonas* sp. was higher than that reported for  $\alpha$ -L-AFases from *Aureobasidium pullulans* (6.99 U/mg) (Saha and Bothast, 1998a), *Streptomyces diastaticus* (10 U/mg and 12.5 U/mg) (Tajana *et al.*, 1992), *Thermomonospora fusca* (0.04 U/mg) (Tüncer, 2000), *Bifidobacterium breve* K-110 (6.3 U/mg) (Shin *et al.*, 2003) and *Aspergillus kawachii* (0.92 U/mg) (Koseki *et al.*, 2003). However,  $V_{max}$  of *Pseudomonas* sp was lower than that reported for  $\alpha$ -L-AFase from *Thermobacillus xylanolyticus* (456 U/mg) (Debeche *et al.*, 2000), *Cytophaga xylanolyticus* (319 U/mg) (Renner and Breznak, 1998) and *B. stearrowthermophilus* T-6 (749 U/mg) (Gilead and Shoham, 1995). On the other hand,  $\alpha$ -L-AFase from *Pseudomonas* sp. showed better affinity for the test substrate than that reported in some  $\alpha$ -L-AFases from other bacteria. This was apparent from the low  $K_m$  value of the enzyme which was lower than that reported for *B. pumilus* (1.7 mM) (Degrassi *et al.*, 2003), *Butyrivibrio fibrisolvens*



**Fig. 3.6a:** Effect of substrate concentration on the activity of  $\alpha$ -L-AFases produced by *Pseudomonas* sp NIOCCAr27.

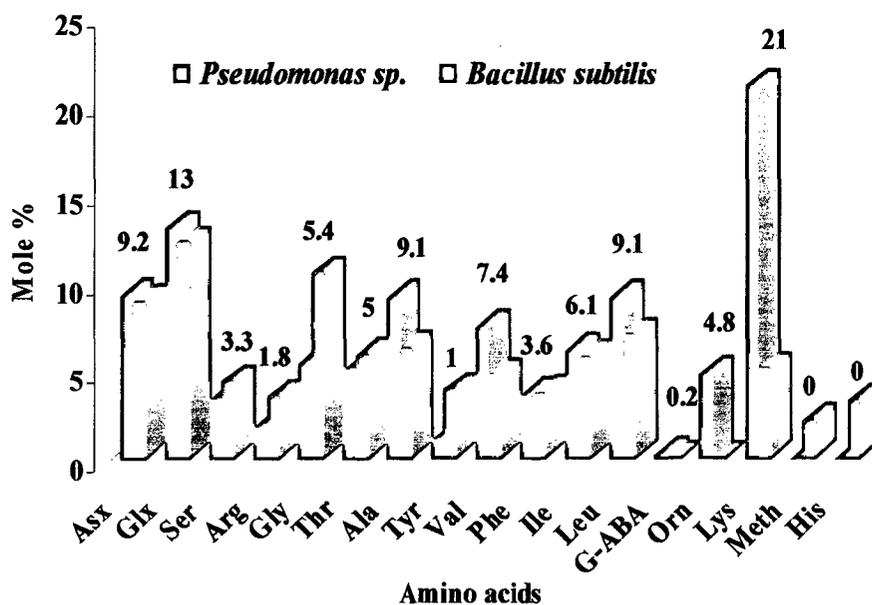


**Fig. 3.6b:** Double reciprocal plot for determination of the  $V_{max}$  and  $K_m$  values of  $\alpha$ -L-AFases against p-nitrophenyl- $\alpha$ -L-arabinofuranoside at 65°C.

(1.04 mM) (Hespell and O'Bryan, 1992), *B. stearothermophilus* T-6 (0.42 mM) (Gilead and Shoham, 1995), *Thermobacillus xylanilyticus* (0.72 mM) (Debeche *et al.*, 2000), *Cytophaga xylanolyticus* (0.504 mM) (Renner and Breznak, 1998), Bacterium PRI-1686 (0.6 mM) (Birigsson *et al.*, 2004), *Closteridium cellulovorans* (0.71 mM) (Kosugi *et al.*, 2002) and *Streptomyces diastaticus* (0.4 mM and 1.25 mM) (Tajana *et al.*, 1992).

### 3.3.8 Amino acid composition

The amino acid composition of  $\alpha$ -L-AFase from *Pseudomonas* sp and *Bacillus subtilis* obtained by HPLC method is presented in Figure 3.7. For comparison purpose the amino acid composition of  $\alpha$ -L-AFase from *B. subtilis* (Kaneko *et al.*, 1994) was also plotted in the same figure (Fig. 3.7). *Pseudomonas* sp  $\alpha$ -L-AFases contained amino acids that are composed of 35.3% nonpolar, 74.7% polar, 22.2% acidic, and 22.8 % basic amino acids. The enzyme contains more basic than acidic residues. However, amino acid compositions of  $\alpha$ -L-AFases from a few reported microbial sources indicated predominantly aspartic acid, glutamic acid, glycine, and threonine (Kaneko *et al.*, 1994; Kimura *et al.*, 1995).



**Fig. 3.7:** Amino acid composition of  $\alpha$ -L-AFase from *Pseudomonas* sp. Amino acid composition of  $\alpha$ -L-AFase from *B. subtilis* (Kaneko *et al.*, 1994) also given for comparison. Numbers in the figure are corresponding to the mole % of each amino acid present in  $\alpha$ -L-AFase from *Pseudomonas* sp.

Amino acid composition of  $\alpha$ -L-AFase from *Pseudomonas sp* showed higher mole ratios for these amino acids compare to  $\alpha$ -L-AFases from *Bacillus subtilis* 3-6 (Kaneko *et al.*, 1994) (Fig. 3.7) and *Aspergillus sojae* (Kimura *et al.*, 1995). Protein functions are governed by a fine balance between the stability and flexibility of proteins. In addition, the role of these factors in biological functions is not well understood (Yamaguchi *et al.*, 2005). Furthermore, stable proteins from mesophiles usually formed by “rigidifying” mutations (Van den Burg *et al.*, 1998; Bogin *et al.*, 1998). These mutated proteins are rigid because of deleting or shortening of loops (Russell *et al.*, 1997), increasing hydrophobic interactions, ion pairs, hydrogen bonds and disulfide bridges (Vieille and Zeikus, 1996; 2001). Furthermore, amino acid composition of the N-terminal of thermophilic endoarabinanase revealed the important of certain amino acids in stability (Yamaguchi *et al.*, 2005). These include glycine and phenylalanine, which were found also in significant amount in  $\alpha$ -L-AFase from *Pseudomonas sp*. However, reasoning the stability to this finding only is not appropriate at this stage. Further investigation for the structural and function relationship is required for confirmation of the role these amino acid in enzyme stability.

### **3.3.9 Effect of Metal ions and other chemical agents**

Chemical factor such as metal ions, reducing agents and metal chelators may induce or inhibit enzyme activity. The activity of  $\alpha$ -L-AFase was assayed in the presence and absence of metal ions, metal chelator (EDTA) and SDS (Table 3.4).  $\text{Cu}^{2+}$  caused partial inhibition of  $\alpha$ -L-AFase activity, whereas complete inhibition was observed in the presnec of  $\text{Hg}^{2+}$  and SDS.

**Table 3.4:** Effect of metal ions and chemical agents on  $\alpha$ -L-AFase activity

Chemical agent	Residual Activity (%)
None	100.0
Ca <sup>2+</sup>	100.4
Co <sup>2+</sup>	97.2
Cu <sup>2+</sup>	77.9
Fe <sup>2+</sup>	100.6
Hg <sup>2+</sup>	13.1
Mg <sup>2+</sup>	99.2
Mn <sup>2+</sup>	98.9
Zn <sup>2+</sup>	91.1
EDTA	100.9
SDS	0.8

Similar inhibitory effect was also observed on  $\alpha$ -L-AFases from *B. stearothermophilus* (Gilead and Shoham, 1995), *Bifidobacterium longum* B667 (Margolles and de los Reyes-Gavián, 2003), *Bacillus thermodenitrificans* (Takao *et al.*, 2002), *Thermobacillus xylanilyticus* (Debeche *et al.*, 2000), *Clostridium cellulovorans* (Kosugi *et al.*, 2002), *Pichia capsulata* X91 (Yanai and Sato, 2000), *Penicillium chrysogenum* (Sakamoto *et al.*, 2003; Sakamoto and Kawasaki, 2003), *Rhodotorula flava* (Uesaka *et al.*, 1978), *Aspergillus sojae* (Oshima *et al.*, 2005), *Aspergillus terreus* (Le Clinche *et al.*, 1997), *Streptomyces thermoviolaceus* (Tsujiibo *et al.*, 2002), *Thermomonospora Fusca* BD25 (Tuncer, 2000), *Penicillium capsulatum* (Filho *et al.*, 1996), *Ruminococcus albus* 8 (Greve *et al.*, 1984), *Aspergillus nidulans* (Fernández-Espinar *et al.*, 1994) and *Sclerotinia sclerotiorum* (Riou *et al.*, 1991). Many authors suggested that inhibition of the enzyme occurs by reacting Hg<sup>+2</sup> ions with free thiols in sulfhydryl groups of the protein (Gilead

protein (Gilead and Shoham, 1995; Tsujibo *et al.*, 2002; Khandeparker *et al.*, 2008). It also reacts with cysteine, histidine and tryptophan residues of the protein. This interaction reduces the molecule's availability for normal metabolic functions (Volkin and Klibanov, 1989). Nevertheless, the OPA reagent used in the present study for amino acid analysis does not react very well with cysteine and histidine. As a result it was not possible to detect the presence of these amino acids in  $\alpha$ -L-AFase from *Pseudomonas* sp. Therefore, with the obtained data it was not possible to identify with certainty the actual reason for the observed inhibitory effect of  $Hg^{+2}$  ions on the enzyme. However, the inhibition of some other enzymes by  $Hg^{+2}$  was observed even though cysteine was not the constituent of total amino acids of these enzymes (Nakamura *et al.*, 1995; Khandeparker and Bhosle, 2006a). Furthermore, it was observed that neither of the other metal ions nor EDTA has an effect on  $\alpha$ -L-AFase activity. This suggested that the enzyme has no requirement for metal ions such as those used in the study.

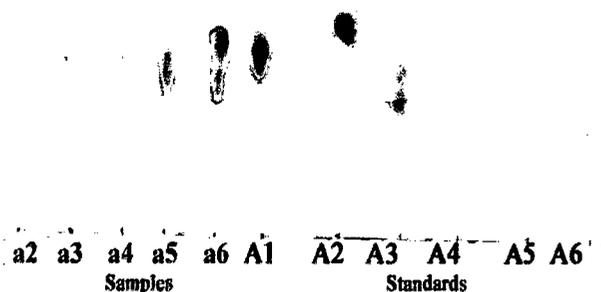
### 3.3.10 Substrate specificity

Comparison of *Pseudomonas* sp  $\alpha$ -L-AFase substrate specificity to other  $\alpha$ -L-AFases reveals relaxed substrate specificity within these enzymes. It has also been shown that within a given class, these enzymes show discrimination between natural and artificial substrates (Beldman *et al.*, 1997). Thus, in order to accurately assign the substrate specificity of  $\alpha$ -L-AFase, a series of artificial and natural substrates were tested.  $\alpha$ -L-AFase was able to cleave the arabinofuranosyl phenyl synthetic substrate, while no activity was detected with the other aryl-glycoside synthetic substrates. The  $\alpha$ -L-AFase from *Pseudomonas* sp exhibits high activity

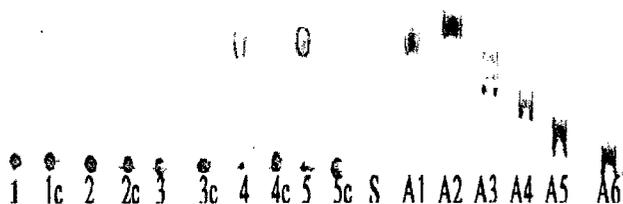
toward pNP- $\alpha$ -L-Araf, but no activity was observed towards pNP $\alpha$ -L-Arap, pNP $\beta$ -L-Arap, pNP $\beta$ -D-Galp, pNP $\beta$ -D-Xylp, pNP $\alpha$ -D-Glcp and pNP $\beta$ -D-Glcp.

With respect to natural substrate,  $\alpha$ -L-AFase from *Pseudomonas sp* was highly active on all arabinofuranooligosaccharides, arabinan and debranched arabinan. The sole hydrolysis product was arabinose as detected by TLC (**Plate 3.2a** and **Plate 3.2b**). The hydrolysis rate of arabinan and debranched arabinan were 78.4% and 59.4% respectively.

With respect to hydrolysis of the synthetic substrate p-nitrophenyl- $\alpha$ -L-arabinofuranose contains the relatively good leaving groups (pKa 7.18). In the natural substrate, however, the leaving groups are either xylose or arabinose moieties, which are very poor leaving groups with pKa's >12 (Wagschal *et al.*, 2007). When hydrolysis of natural substrates glycosidic bonds by  $\alpha$ -L-AFase was tested, it was found that  $\alpha$ -L-AFase released arabinose from sugar beet arabinan containing (1-3)- $\alpha$ -linked L-arabinofuranosyl branch units. Furthermore, the activity was detected on the corresponding debranched 1,5- $\alpha$ -L-linked arabinan polymer as well. However, the fact that over 50% of the branched arabinan could be hydrolyzed and that TLC analysis (**Plate 3.2b**) showed that only arabinose was being released (no arabinobiose or higher degree oligomers), indicates that  $\alpha$ -L-AFase degrades the 1,3-linkages in the side chains as well. On the other hand, all reported  $\alpha$ -L-AFases that have been tested on branched arabinan and arabinan stripped of their side chains (debranched or linear) are found to be much more active on branched arabinan (Gilead and Shoham, 1995; Debeche *et al.*, 2000; Gomes *et al.*, 2000; Degrassi *et al.*, 2003).



**Plate 3.2a:** TLC analysis of the hydrolysis products released from arabinofuranooligosaccharides by *Pseudomonas* sp.  $\alpha$ -L-AFase. Standards include: A1: arabinose, A2: arabinobiose, A3: arabinotriose, A4: arabinotetraose, A5: arabinopentaose and A6: arabinohexaose. Samples include: a2:arabinobiose, a3: arabinotriose; a4: arabinotetraose, a5: arabinopentaose and a6: arabinohexaose.



**Plate 3.2b:** TLC analysis of the hydrolysis products released from arabinofuranooligosaccharides natural substrates by *Pseudomonas* sp.  $\alpha$ -L-AFase. The substrates included: 1:Wheat arabinoxylan, 2: Oat spelt xylan, 3: Arabinogalactan, 4: Debranched arabinan and 5: Arabinan. The Standards: A1: arabinose, A2: arabinobiose, A3: arabinotriose, A4: arabinotetraose, A5: arabinopentaose, A6: arabinohexaose. The controls: 1c:Wheat arabinoxylan, 2c: Oat spelt xylan, 3c: Arabinogalactan,4c: Debranched arabinan and 5c: Arabinan.

$\alpha$ -L-AFase from *Pseudomonas* sp. was almost equally active on both branched arabinan and debranched arabinan. This behavior of  $\alpha$ -L-AFase is in accord with previous study where it has been shown that  $\alpha$ -L-AFase was equally active on both arabinan and debranched arabinan (Birgisson *et al.*, 2004). In addition, no

hydrolysis products were detected for the substrates such as birchwood xylan and beechwood xylan. This is not surprising since  $\alpha$ -L-AFase appears unable to hydrolyze xylopyranoside linkages. Furthermore, it has been reported that these hardwood xylans contain little or no arabinofuranosyl substitution (Timell, 1964). Moreover, no hydrolysis products were detected for larchwood (+)-arabinogalactan (**Plate 3.2b**) even though the ratio of arabinose:galactose units has been reported to be 17:83 for this substrate (Kormelink and Voragen, 1993). These results suggested that  $\alpha$ -L-AFase has no ability to release arabinose from arabinogalactan since arabinose in larchwood (+)-arabinogalactan is mainly O-2 linked to galactose (Kormelink and Voragen, 1993). Surprisingly, no hydrolysis products were detected for wheat arabinoxylan and oat spelt xylan (soluble and nonsoluble) (**Plate 3.2b**). These results are in accord with some previous studies where it has been shown that  $\alpha$ -L-AFases usually have a narrow range of substrate specificity (Beldman *et al.*, 1997; Van Laere *et al.*, 1997; Miyazaki, 2005).

With respect to natural substrate specificity, it can be concluded that  $\alpha$ -L-AFase is specific for hydrolyzing arabinofuranosyl units attached to arabinose moieties. These results further suggest that  $\alpha$ -L-AFase may be specific for hydrolysis of (1-3)- $\alpha$ -L- and (1-5)- $\alpha$ -L- arabinofuranosyl branch units present in arabinan and debranched arabinan. Further,  $\alpha$ -L-AFase activity on arabinans and debranched arabinans only may suggest that the substrate specificity of  $\alpha$ -L-AFase was dependent on the nature of the aglycone component of the substrate. These results are in accord with previous study where in  $\alpha$ -L-AFase from *Thermotoga maritima* was active only on arabinan and debranched arabinan (Miyazaki, 2005). However,

the rate of hydrolysis of arabinan and debranched arabinan by this enzyme was 20:1, implying that the enzyme cleaves the  $\alpha$ -1,2- or  $\alpha$ -1,3-linkage much more efficiently than  $\alpha$ -1,5-linkage (Miyazaki, 2005). Moreover, exo-arabinanase (Sakamoto and Thibault, 2001) and endo-arabinanase (Takao *et al.*, 2002; Fontes Leal and de Sá-Nogueira, 2004) may show activity on arabinan and debranched arabinans. However, the hydrolysis products of these enzymes include arabinobiose or arabinotriose and not arabinose. Furthermore, both types of these enzymes have no activity on arabinobiose and p-nitrophenyl- $\alpha$ -L-arabinofuranoside (Sakamoto and Thibault, 2001, Takao *et al.*, 2002).

To determine whether  $\alpha$ -L-AFase is an endo- or exo-acting enzyme, the products formed at different reaction times were monitored with TLC using debranched arabinan as the substrate. Generally, in the case of exo-acting enzymes, only monomers or dimers accumulate throughout the enzyme reaction, while in the case of endo-acting enzymes, larger oligomers form even in the initial stage of hydrolysis (Sakamoto *et al.*, 2003). In the case of  $\alpha$ -L-AFase from *Pseudomonas* sp only arabinose and no arabinobiose or larger oligomers were detected in the reaction mixture. This provides clear evidence that  $\alpha$ -L-AFase from *Pseudomonas* sp released arabinose by an exo-acting fashion.

It was clear that  $\alpha$ -L-AFase from *Pseudomonas* sp is an exo-acting enzyme which exhibits a high degree of specificity for the  $\alpha$ -L-arabinofuranosyl moiety of arabinan and debranched arabinan. The enzyme is not tolerant to other different aglycone moieties. Most importantly,  $\alpha$ -L-AFase could be distinguished from several  $\alpha$ -L-arabinofuranosidases in the ability to hydrolyze arabinan, debranched

arabinan and p-nitrophenyl- $\alpha$ -L-arabinofuranoside. A thorough description of the substrate specificity, and hence biological function, of the  $\alpha$ -L-AFase will depend on the determination of the three-dimensional structure of the enzyme. The properties of this enzyme could be of benefit if utilized for degradation of arabinans that cause haze formation during production of fruits juices and for production of L-arabinose - the antiglycemic sugar- from arabinans containing substrates.

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# *Chapter 4*

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Optimization of Growth Medium and Conditions for  
 $\alpha$ -L-arabinofuranosidase Production by *Bacillus* sp NIOCCW19  
grown under Solid-State Fermentation and Submerged Fermentation

#### 4.1.1 INTRODUCTION

Choosing the appropriate cultivation system is an important criterion that should be considered for economic production of industrial enzymes. Furthermore, the cost-effectiveness of the medium used for enzymes production is an important factor that has to be considered while developing a production medium. This can be achieved by using cheaply available agricultural residues such as wheat bran, oat bran, sugar cane bagasse, rice husks, etc. Therefore, it was essential to assess  $\alpha$ -L-arabinofuranosidase ( $\alpha$ -L-AFase) production by *Pseudomonas* sp NIOCCAr27 using agricultural residues under solid state fermentation (SSF) and submerged fermentation (SmF). *Pseudomonas* sp failed to grow under SSF condition and produces low amounts of  $\alpha$ -L-AFase when grown on cheap agricultural residues under SmF. Nevertheless, this bacterium produces  $\alpha$ -L-AFase with desirable properties that has not been reported before. However, the enzyme was active against few substrates which included arabinan and debranched arabinan. An enzyme with broader substrate specificity which can be produced at low cost would be more desirable for industrial applications. Because of this, another bacterial isolate *Bacillus* sp NIOCCW19 was selected for further studies. This culture produces good amounts of  $\alpha$ -L-AFase and xylanase when grown on cheap agricultural residues under SmF and SSF (Table 2.4, Chapter 2). SmF and SSF are well known cultivation systems that have been used for enzymes production from bacteria and fungi. SmF has advantages in process control and easy recovery of extracellular enzymes.

However, the products are dilute and enzymatic extracts might be less stable than those from SSF. SSF has the advantages of simplicity, lower production costs, lower energy expenditures, lower risk of contamination, high enzyme yields and low effluents output (Pandey, 1992; Pandey *et al.*, 1999; 2000; 2001; Pandey, 2003; Couto and Sanromán; 2006). However, problems such as temperature and pH control are encountered in SSF and some reports indicated that there is no difference in enzymes yields between SSF and SmF (Ramamurthy and Kothari, 1993; Smits *et al.*, 1996; Nagel *et al.*, 2001; dos Santos *et al.*, 2004). Studies on production of  $\alpha$ -L-AFase from bacteria were restricted to the use of SmF. Most of these studies, however, are dealt with  $\alpha$ -L-AFases purification and characterization, with only one report regarding their production optimization (Gomes *et al.*, 2000). Furthermore, in all of these studies; less attention was made to the effects of cultivation system type and fermentation variables on  $\alpha$ -L-AFase production. Therefore, optimization of such fermentation variables has to be carried to define the most suitable conditions for maximum enzyme yields in SSF and SmF. Within this context, the present investigation aimed to: contribute towards understanding the effect of fermentation variables on  $\alpha$ -L-AFase production by *Bacillus* sp NIOCCW19 through SSF and SmF in order to define optimum medium components and growth conditions for maximum  $\alpha$ -L-AFase production and to comparatively evaluate the enzyme yield in SSF and SmF.

## **4.1.2 MATERIALS and METHODS**

### **4.1.2.1 Microbial strain**

*Bacillus* sp NIOCCW19 used in the present investigation was isolated from sediment sample that was collected from Mandovi estuary Goa, India (Table

**2.6, Chapter 2).** The strain was maintained on Zoble marine agar (ZMA) (Himedia, Mumbai, India) slants at 4°C. It was sub-cultured every 2 weeks by growing in the same medium at 30 °C for 48 h.

#### **4.1.2.2 Preparation of inoculum**

To prepare inoculum, the *Bacillus* sp NIOCCW19 was grown in MBSM supplemented with birchwood xylan (Sigma) as a carbon source. MBSM composition is described in **Table 2.1** of **chapter 2**. Medium pH was adjusted to pH, 8.00 with 1N NaOH and sterilized at 121°C for 20 min. A loop full of 24 h grown bacterium on ZMA plate was used to inoculate the sterilized medium. Inoculated medium was incubated at 30°C on a rotary shaker (100 RPM) for three days. The bacterial growth was monitored by measuring the O.D. of growth medium at 600 nm. The *Bacillus* sp growth reached a stationary phase after 48 h and has O.D. of 2.88. The stationary phase grown inocula were then used in all the following experiments.

#### **4.1.2.3 Effect of carbon sources on $\alpha$ -L-AFase production under SmF**

Preliminary experiments were conducted to assess the effect of various carbon sources on  $\alpha$ -L-AFase production by *Bacillus* sp grown under SmF. The bacterium was grown in MBSM (as described in **Chapter 2**) at 30°C. Either of birchwood xylan, beechwood xylan, arabinogalactan, arabinan, D-glucose, D-galactose, D-fructose, D-mannose, D-xylose, D-arabionse, L-arabinose, D-sucrose, D-maltose, lactose, cellobiose, inositol, D-manitol, D-sorbitol and L-arabitol and the agricultural substrates including rice husk, wheat bran, sugar cane bagasse, oat bran, oat spelt and sugar beet pulp was used as carbon source

(0.5%) individually in SmF medium. After 96 h growth of the culture on each carbon source,  $\alpha$ -L-AFase activity was estimated.

#### **4.1.2.4 Effect of carbon sources on $\alpha$ -L-AFase production under SSF**

Preliminary experiments were conducted to assess the effect of agricultural substrates on  $\alpha$ -L-AFase production by *Bacillus* sp grown under SSF. The bacterium was grown under SSF at 30°C (as described in Chapter 2). The agricultural substrates moistened with MBSM (substrates to moisture ratio 1:3, w:v) were used individually in SSF medium. After 96 h growth of the culture on each agricultural substrate,  $\alpha$ -L-AFase activity was estimated.

#### **4.1.2.4 Optimization of growth medium and conditions for $\alpha$ -L-AFase production under SSF**

Various process variables were assessed to evaluate their influence on  $\alpha$ -L-AFase production by *Bacillus* sp in SSF. Wheat bran was used as a sole source of carbon in these studies. SSF was carried out in 50 ml Erlenmeyer flasks using 1g of wheat bran moisturized with 3 ml of MBSM. SSF variables studied were incubation temperature (25, 30, 35, 40, 45, 50°C), pH (5,6,7,8, 9,10), initial moisture level of the substrate (1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5), inoculum size (1.0, 1.5, 2.0, 2.5 and 3.0 ml of bacterial suspension). Major nutrient components of MBSM were also studied for their effect on  $\alpha$ -L-AFase production in SSF. These included (mg/ml): inorganic nitrogen  $\text{NH}_4\text{Cl}$  (0; 0.5; 1.25; 2.25; 4.5; 9), inorganic phosphates ( $\text{K}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ ) (0; 0.25; 0.5; 1; 2; 4), peptone (0; 0.25; 0.5; 1; 2; 3) and yeast extract (0; 0.5; 1; 2; 4; 8; 12; 20). In addition, the effect of NaCl % (0; 0.5; 1; 1.5; 2; 2.5; 3; 3.5; 4) on enzyme production was also studied. After five days of growth the

fermented matter was mixed thoroughly with Na-phosphate buffer (50 mM, pH 8.0). The flask containing mixture was placed on rotary shaker 200 RPM, for 30 min at 30 °C. The flask content was centrifuged (10,000 RPM) for 10 min at 4°C and the supernatant was used for enzyme estimation. The SSF and SmF optimized medium obtained after conducting these experiments is called hereafter SSF and SmF Modified Basal Salt Medium No.2 (SSF and SmF MBSM2). The composition of SSF and SmF-MBSM2 is given in **Table 4.1.1**.

**Table 4.1.1:** The composition of the SSF and SmF Modified Basal Salt Medium No.2 (SSF and SmF-MBSM2).

Chemical composition	SSF	SmF
	per liter	per liter
NaCl	25.00 g	25.00 g
KCl	0.75 g	0.75 g
MgSO <sub>4</sub>	7.00 g	7.00 g
NH <sub>4</sub> Cl	2.250 g	2.250 g
K <sub>2</sub> HPO <sub>4</sub> (10%)	7.00 ml	7.00 ml
KH <sub>2</sub> PO <sub>4</sub> (10%)	3.00 ml	3.00 ml
Yeast extract	2.00 g	4.00 g
Peptone	0.50 g	1.00 g
Trace metals solution	1.00 ml	1.00 ml
Distilled water	1000 ml	1000 ml
pH adjusted with 1N NaOH	pH 8.0	pH 8.0

#### 4.1.2.5 Optimization of growth medium and conditions for $\alpha$ -L-Afase production under SmF

Different process variables were assessed to evaluate their influence on  $\alpha$ -L-Afase production in SmF. SmF variables studied were those described above in SSF except for moisture content as it is not applicable in this case.

Wheat bran was used as a sole source of carbon in these studies. SmF was

conducted in 50 ml Erlenmeyer flasks using 2% wheat bran in 10 ml MBSM. After five days of growth period, the fermented medium was centrifuged (10,000 RPM) for 10 min at 4°C and collected supernatant was used for enzymes estimation.

#### **4.1.2.6 Growth and enzyme production under solid-state fermentation**

*Bacillus* sp cells grown for 48 h were used to inoculate 250 ml Erlenmeyer flasks containing 30 ml of SSF-MBSM2 (Table 4.1.1) and 10 g (substrates to moisture ratio 1:3) of either of agricultural substrates (rice husk, wheat bran, sugar cane bagasse, oat bran, oat spelt and sugar beet pulp). The flasks were autoclaved at 121°C for 20 min and then cooled to room temperature. After cooling, medium was inoculated with 1ml of bacterial inoculum. The inoculated flasks were incubated at 40°C under static conditions. Flasks were gently tapped intermittently to mix the contents. SSF was conducted for six days using a set of seven flasks for each substrate used. To assess growth (measured as cell protein) and enzyme production under SSF, one flask was removed at the desired intervals. The fermented matter was processed as describe above. The resulted supernatant was used for enzyme activity estimation. The resulted pellets were washed several times with Na-phosphate buffer and centrifuged (10,000 RPM) for 10 min at 4°C. The pellets were then oven dried at 80°C for 48 h and used for bacterial protein determination.

#### **4.1.2.7 Growth and enzyme production under submerged fermentation**

The culture was growing under SmF by taking 50 ml of SmF-MBSM2 (Table 4.1.1) in 250 ml Erlenmeyer flasks. This was supplemented individually with various agricultural substrates at 2% concentration. The pH of the medium was

adjusted to pH 8.0 using 1N NaOH. All the flasks were autoclaved at 121 °C for 20 min then cooled to room temperature. After cooling, media were inoculated with 3 ml of bacterial inoculum pre-grown for 48 h. Flasks containing inoculated media were incubated at 40°C on a rotary shaker at 100 RPM. SmF was conducted for six days using a set of seven flasks for each substrate used. To assess growth (measured as cell protein) and enzymes production under SmF, one flask was removed at the desired intervals and the fermented matter was processed as describe above.

#### **4.1.2.8 Analytical method**

##### **Enzyme assay**

$\alpha$ -L-AFase activity in the supernatants of SSF and SmF fermented media was estimated as described in chapter 2. Enzyme estimation was conducted at 75°C and Na-phosphate buffer (50 mM, pH 8.0) was used for dissolving the chromogenic substrate *p*-NPAF. One unit (U) of  $\alpha$ -L-AFase activity was defined as the amount of enzyme that librated 1 $\mu$ mol of *p*NP at 75°C in 1 min.  $\alpha$ -L-AFase production in SmF and SSF were expressed as units (U) per gram (g) of initial dry substrate (IDS) (U/g IDS) following Acuña-Argüelles *et al.* (1995) and Sandhya *et al.* (2005).

##### **Bacterial biomass estimation**

Bacterial biomass was estimated as bacterial cell protein and determined according to the method of Khandeparker and Bhosle (2006a). For that, cells were treated with 0.1 N NaOH in the boiling water bath for 30 min so as to extract proteins. Sample was cooled and neutralized using 1N HCl. A suitable aliquot was then used to estimate protein using the method of bicinchoninic acid

(Smith *et al.*, 1985). Bacterial biomass protein production in SmF and SSF were expressed as mg per gram of initial dry substrate (mg/g IDS).

Comparing enzyme titers and bacterial biomass production in SSF and SmF was done according to Acuña-Argüelles *et al.* (1995) and Sandhya *et al.* (2005).

#### **4.1.3 RESULTS AND DISCUSSION**

##### **Effect of carbon sources on $\alpha$ -L-AFase production under SSF and SmF**

*Bacillus* sp was grown on different agricultural residues under SSF. Only a few reports relate the use of some of these agricultural residues as substrates for  $\alpha$ -L-AFase production in SSF by fungi (Roche *et al.*, 1995; Filho *et al.*, 1996). To the best of our knowledge, there is only one report on the production of  $\alpha$ -L-AFase in SSF by bacteria (Khandeparker *et al.*, 2008). Furthermore, the culture was also grown under SmF condition using the agricultural residues and the soluble carbon sources that included arabinose containing polymers, sugars and sugar alcohols. The culture was able to grow under both culturing systems on all carbon sources used. This suggests that the culture has high enzymatic potentials which enable it to grow in simple and complex substrates. The enzyme production was influenced by both the carbon sources and the cultivation systems used for growing the bacterium (Tables 4.1.2 and 4.1.3). This finding was in agreement with previous report that carbon source and cultivation system effects  $\alpha$ -L-AFase production by bacteria (Khandeparker *et al.*, 2008). Furthermore, the differences in enzyme yields could be attributed to the wide variations in the chemical structure of substrates used (Timell, 1964,1967; Fincher and Stone, 1986). Among the sugars and sugar alcohols used as a carbon sources for  $\alpha$ -L-AFase production, L-arabinose, D-arabinose,

alcohols used as a carbon sources for  $\alpha$ -L-AFase production, L-arabinose, D-arabinose, D-xylose and L-arabitol were the best inducer compare to other sugars used (Table 4.1.3). All of these carbon sources were reported to be good inducers for  $\alpha$ -L-AFase production in some bacteria and fungi (Kaji *et al.*, 1967;1981; Komae *et al.*, 1982; Beldman *et al.*, 1997; Saha, 2000).

**Table 4.1.2:** Effect of agricultural residues on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown under SSF and SmF.

Carbon source	$\alpha$ -L-AFase (U/g IDS) SSF	$\alpha$ -L-AFase (U/g IDS)SmF
Wheat bran	14.94	3.68
Oat bran	11.35	3.91
Sugar cane bagasse	5.97	0.99
Sugar beet pulp	4.04	0.817
Rice husks	0.914	0.126
Oat spelt xylan	7.92	2.47

**Table 4.1.3:** Effect of arabinose containing polymers, sugars and sugar alcohols on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown under SmF.

Carbon source	$\alpha$ -L-AFase (U/ml)
Birch wood xylan	0.371
Beech wood xylan	0.428
Arabinan	0.136
Arabinoglactan	0.009
L-arabinose	2.14
D-arabinose	1.43
D-xylose	1.25
D-glucose	0.033
Galactose	0.045
Fructose	0.022
Mannose	0.055
Maltose	0.092
Sucrose	0
Lactose	0
D-sorbitol	0.099
D-mannitol	0
Cellobiose	0
L-arabitol	0.614
Inositol	0.002

The amount of enzyme produced by using pentose sugars and sugar alcohols was higher than that reported from many thermophilic and mesophilic bacteria such as *Thermotoga thermarum* (Sunna *et al.*, 1996), *Bacillus subtilis* (Poutane *et al.*, 1987), *Bacillus stearothermophilus* (Gilead and Shoham, 1995), *Rodothermus marinus* (Gomes *et al.*, 2000), *Bacillus pumilus* PS213 (Degrassi *et al.*, 2003) and *Arthrobacter* sp (Khandeparker *et al.*, 2008). The  $\alpha$ -L-AFase production by the bacterium was also higher than that reported in many fungi such as *Aspergillus awamori*, *Fusarium oxysporium*, *Aspergillus niger* (Poutane *et al.*, 1987), *Aureobasidium pullulans* (Saha and Bothast, 1998a) and *Aspergillus terreus* (Clinnche *et al.*, 1997).

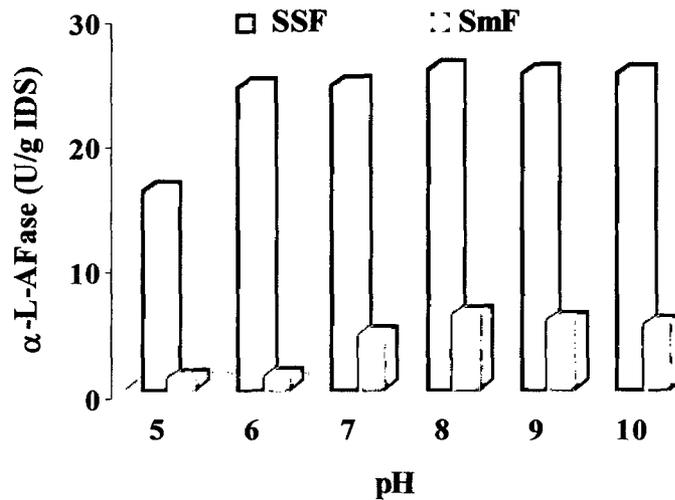
The production of  $\alpha$ -L-AFase using agricultural residues showed the highest yields when wheat bran and oat bran were used (Table 4.1.2). In fact, these two cereal substrates contain softwood arabinoxylan, which is usually hydrolyzed by xylanolytic enzymes that include  $\alpha$ -L-AFase (Saha, 2000; Khandeparker *et al.*, 2008). Comparing the enzyme yields obtained by growing the bacterium on agricultural residues with that from other bacteria was difficult. This was due to the difference in the cultivation system and nature of carbon sources used with other bacteria. In general,  $\alpha$ -L-AFase activity observed from wheat barn and oat bran was greater than that reported for other bacteria grown under SmF on some arabinose containing substrates. These included actinomycetes such as *Streptomyces* no-17-1 (Kaji *et al.*, 1981) *Streptomyces diastatochromogenes* 065 (Higashi *et al.*, 1983), *Streptomyces* spp (Johnson *et al.*, 1988), *Streptomyces* strains (Zimmerman *et al.*, 1988) and *Streptomyces diastaticus* (Tajana *et al.*, 1992). The enzyme production using these substrates was also greater than that reported for bacterial strains such as *Bacillus macerans*

(William and Withers, 1985), *Bacillus stearothermophilus* (Gilead and Shoham, 1995), *Cytophaga xylanolytica* (Renner and Breznak, 1998) and *Bacillus pumilus* ( Degrassi *et al.*, 2003). The ability of *Bacillus* sp NIOCC-W19 to grow on agricultural residues and produce high amount of  $\alpha$ -L-AFase may suggest that it is a good culture for large-scale production of  $\alpha$ -L-AFase using these rather inexpensive carbon sources.

### **Influence of initial pH on enzyme production in SmF and SSF**

Enzymes production by microbial strains strongly depends on the extracellular pH. This is because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and enzymes production (Moon *et al.*, 1991; Ellaiah *et al.*, 2002; Prakasham *et al.*, 2006). The effect of initial pH on  $\alpha$ -L-AFase production by *Bacillus* sp when grown on wheat bran under SSF and SmF conditions is presented in **Figure 4.1.1**. The maximum  $\alpha$ -L-AFase production was observed at pH 8.0 in SmF medium. However, further increase in pH showed no significant effect on the enzyme production. The enzymes production reduced significantly in SmF medium with pH lower than 7.0. In SSF medium, however, pHs below pH 6.0 resulted in slight decrease in enzymes production (**Fig. 4.1.1**). On the other hand, no significant difference in enzyme production was observed in SSF medium when the pH was varied from 6.0 to 10 (**Fig.4.1.1**). Similar observations were found for protease production by *Bacillus* sp strain S4 and *Pseudomonas* sp strain S22 in wheat bran SSF and SmF based media (Adesh *et al.*, 2002). The phenomenon of pH unaffected enzymes production in wheat bran based-SSF medium could be explained by the fact that wheat bran possesses excellent buffering capacity (as also some

other agro-industrial residues) (Pandey *et al.*, 1999; 2001; Sandhya *et al.*, 2005). Moreover, it was reported that different mechanisms regulate the transcription of genes coded for enzymes which are controlled by SSF and SmF-specific environmental conditions. The control mechanism in such case could be pH dependent, involving a pH-dependent transcription regulator (Te Biesebeke *et al.*, 2005).



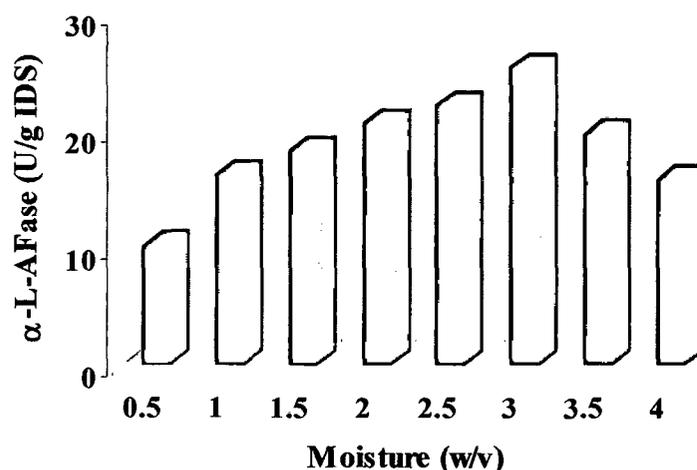
**Fig. 4.1.1:** Effect of initial pH on  $\alpha$ -L-AFase production by *Bacillus* sp grown in wheat bran based-SSF and SmF media.

Moreover, study at the molecular level revealed that  $\alpha$ -L-AFase synthesis by *Aspergillus niger* was controlled by pH of SmF liquid growth medium. In the acidic pH, the expression of *abfB* gene coded for  $\alpha$ -L-AFase was higher than that obtained in neutral or alkaline pH (Gielkens *et al.*, 1999).

#### **Influence of incubation temperature on enzyme production in SSF & SmF**

The significance of temperature in the development of a biological process is such that it could determine the effects of protein denaturation, enzymatic inhibition, promotion or suppression of the production of a particular metabolite, cell viability and death (Pandey *et al.*, 2001). The effect of incubation temperature on  $\alpha$ -L-AFase production in SSF and SmF media is

yield (Feniksova *et al.*, 1960; Nishio *et al.*, 1979; Ramesh and Lonsane, 1990; Ramachandran *et al.*, 2004; Prakasham *et al.*, 2006). This is because growth of microbes and product formation takes place at or near the surface of moist solid substrate (Pandey *et al.*, 2000). Therefore, for achieving maximum yield of the desirable product, it is most crucial step to optimize the moisture content that controls the water activity ( $a_w$ ) of the fermenting substrate (Prakasham *et al.*, 2006). However, this factor is not applicable to liquid medium (SmF). The result of the study presented in **Figure 4.1.3** showed that the  $\alpha$ -L-AFase yield was optimum with (1:3 w/v) moisture level. This was higher than that found in earlier reports, which described the requirement of (1:2.5 w/v) and (1:1 w/v) initial moisture content for maximum production of extracellular xylanolytic enzymes by *Bacillus licheniformis* A99 (Archana and Satyanarayana, 1997) and *Bacillus* sp AR-009 (Gessesse and Mamo, 1999), respectively. However, similar initial moisture content was optimal for the production of  $\alpha$ -L-AFase and xylanase from *Arthrobacter* sp (Kanderparker and Bhosle, 2006a; Kanderparker *et al.*, 2008) and xylanase from *Streptomyces* sp QG-11-3 (Beg *et al.*, 2000).



**Fig. 4.1.3:** Effect of initial moisture on  $\alpha$ -L-AFase production by *Bacillus* sp grown in wheat bran based-SSF medium.

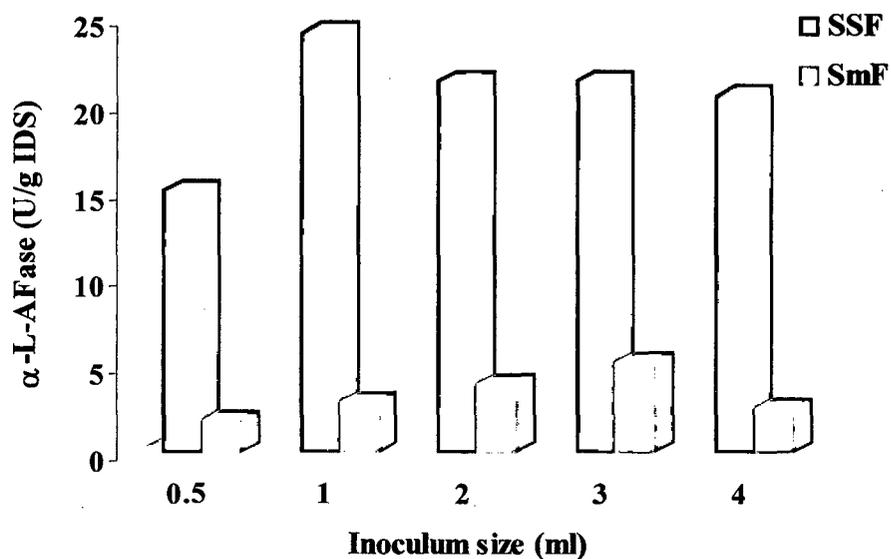
Furthermore, higher initial moisture content (1:6) was optimal for the production of extracellular xylanolytic enzyme by *Bacillus coagulans* BL69 (Heck *et al.*, 2005). On increased addition of moisture to the medium, enzyme production was reduced. At lower and higher initial moisture levels than the optimum affects the metabolic activities of the bacteria and consequently enzymes synthesis also affected.

The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of the solid substrate (Feniksova *et al.*, 1960; Raimbauh and Alazard, 1980; Narahara *et al.*, 1982). A higher than optimum moisture level causes decrease in porosity, alteration in wheat bran particle structure, gummy texture, reduction in gas volume and lower oxygen transfer (Feniksova *et al.*, 1960; Raimbauh and Alazard, 1980; Lekha and Lonsane, 1994). In contrast, a lower moisture level than the optimum leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling and a higher water tension (Feniksova *et al.*, 1960; Zandrazil and Brunert, 1981). The fact that the bacterium grows and produces maximum  $\alpha$ -L-AFase activities at low wheat bran-to-moisture ratio and at an alkaline pH will offer significant advantage in reducing the risk of contamination. This is because most bacterial species are unable to grow at reduced moisture level as well as in alkaline pH.

#### **Effect of inoculum size for enzyme production in SSF and SmF**

Size of inoculum is an important biological factor, which determines biomass production in fermentation (Sandhya *et al.*, 2005). **Figure 4.1.4** shows that there was a significant increase in  $\alpha$ -L-AFase production with the increase in inoculum size up to the maximum level after that enzyme yield was reduced.

Maximum  $\alpha$ -L-AFase production was observed when SSF medium inoculated with 1 ml of the bacterial suspension (10% v/w). However, maximum enzyme yield was observed when 50 ml SmF medium was inoculated with 3 ml of bacterial suspension. Lower inoculum levels than the optimum resulted in lower enzyme yield, whereas, larger inoculum levels than the optimum reduced enzyme yield in SSF and SmF media (Fig. 4.1.4). This is because a lower inoculum density may give insufficient biomass and permit the growth of undesirable organisms (Selvakumar and Pandey, 1999). Moreover, the decrease observed in enzyme production when larger inoculum sizes were used could be due to the shortage of nutrients available for the larger biomass and faster growth of the culture (Hesseltine *et al.*, 1976; Selvakumar and Pandey, 1999; Sandhya *et al.*, 2005). Thus a balance between the proliferating biomass and available material should be maintained for maximum enzyme production (Sandhya *et al.*, 2005).



**Fig. 4.1.4:** Effect of inoculum size on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in wheat bran based-SSF and SmF media

### Influence of nitrogen sources on enzyme production in SSF and SmF

The maximum  $\alpha$ -L-AFase yields was obtained when SSF and SmF media were supplemented with (2.25 mg/ml) of  $\text{NH}_4\text{Cl}$ . The absence of inorganic nitrogen source decreased the yield of  $\alpha$ -L-AFase by 36.1% when compared to maximum enzyme yield obtained with the optimum concentration of  $\text{NH}_4\text{Cl}$  (Fig. 4.1.5).

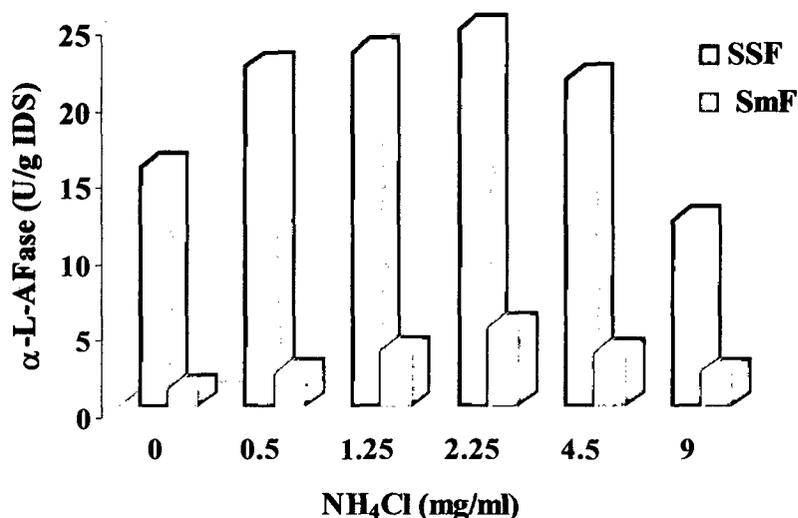
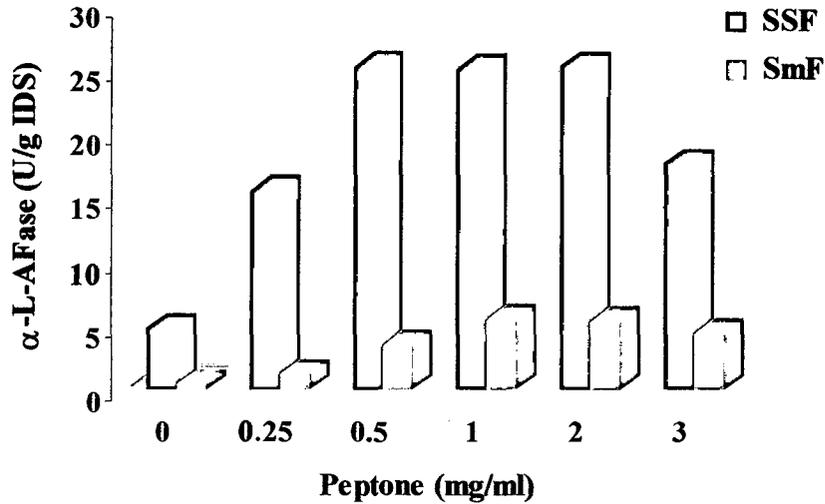


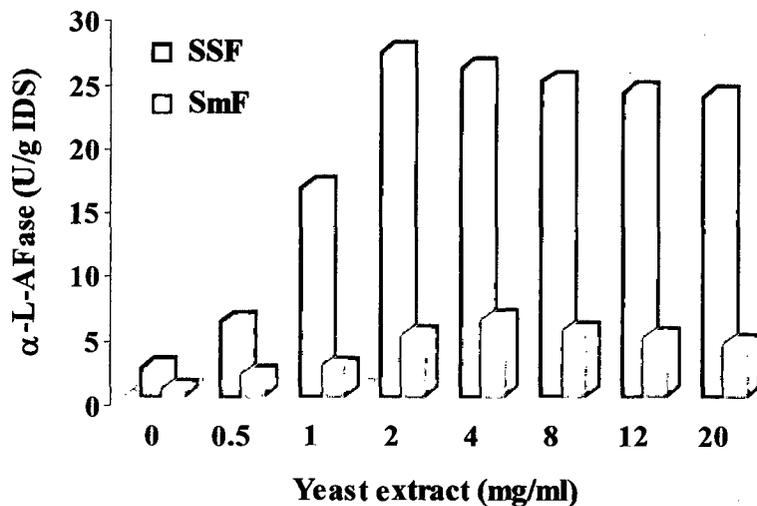
Fig. 4.1.5: Effect of  $\text{NH}_4\text{Cl}$  concentration on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in wheat bran based-SSF and SmF media.

The maximum  $\alpha$ -L-AFase yield in SmF medium was observed in media with high concentrations of peptone (1 mg/ml) and yeast extract (4 mg/ml) (Fig. 4.1.6 and Fig. 4.1.7). In contrast, lower concentrations of peptone (0.5 mg/ml) and yeast extract (2 mg/ml) were required for maximum production of  $\alpha$ -L-AFase in SSF medium (Fig. 4.1.6 and Fig. 4.1.7). Previous studies on xylanolytic enzymes production by *Thermomyces lanuginosus* under SSF and SmF have shown yeast extract as critical nitrogen source (Hoq *et al.*, 1994; Singh *et al.*, 2000). The importance of the nitrogen source has come from the fact that, the degree of selectivity for the production of a particular enzyme may be regulated by the carbon to nitrogen ratio (Shah and Madamwar, 2005). For

example, by limiting the available sources for protein synthesis under low nitrogen to carbon ratio, a more strict separate regulation of the synthesis of xylanolytic and cellulytic enzymes occurs in some microorganisms (Bailey *et al.*, 1991; Gerber *et al.*, 1997, Shah and Madamwar, 2005).



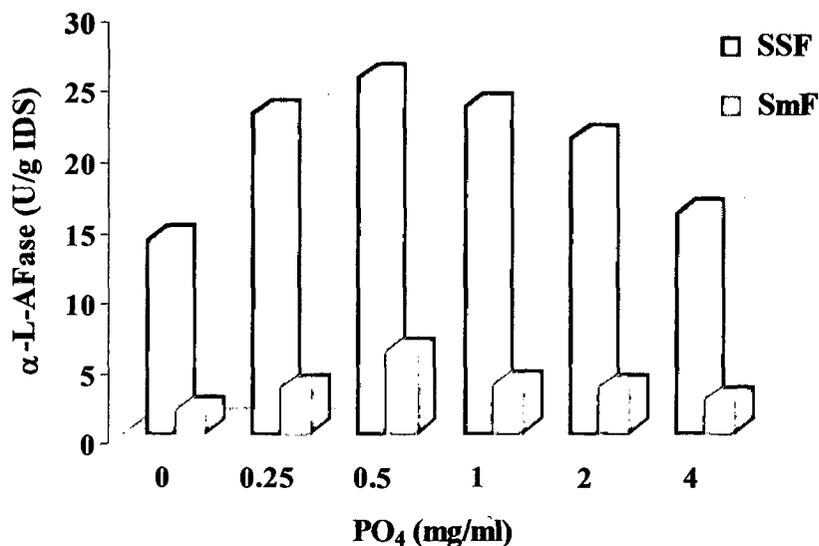
**Fig. 4.1.6:** Effect of peptone concentration on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in wheat bran based-SSF and SmF media.



**Fig. 4.1.7:** Effect of yeast extract concentration on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in wheat bran based-SSF and SmF media.

### Influence of phosphate on enzyme production in SSF and SmF

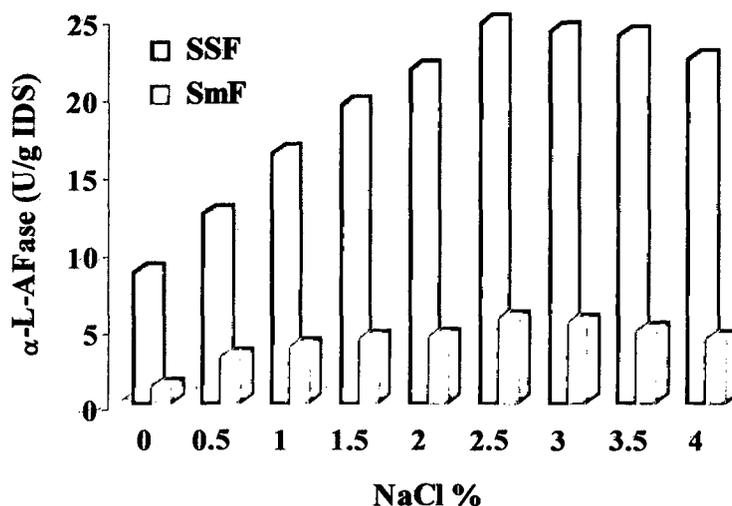
The presence of phosphate has a great importance for optimization of enzymes production by microorganism (Pinto *et al.*, 2003). **Figure 4.1.8** shows the effect of phosphate concentration on  $\alpha$ -L-AFase production by *Bacillus* sp grown in wheat bran based-SSF and SmF media. The maximum  $\alpha$ -L-AFase yields were obtained in SSF and SmF media supplemented with 0.5 mg/ml of phosphate sources. Furthermore, an increase in phosphate concentration above the optimum concentration caused a reduction in  $\alpha$ -L-AFase yields in SSF and SmF media (**Fig. 4.1.8**). Shoham *et al.* (1992) described the sever reduction in  $\alpha$ -L-AFase production by *Bacillus stearothermophilus* strain T-6 in SmF growth medium with high phosphate concentration. They also reported inhibition of bacterial growth in the presence of 10 mM phosphates (Shoham *et al.*, 1992). Furthermore, according to Pinto *et al.* (2003) using optimum concentration of phosphate promotes increase in the extracellular enzyme synthesis level and a very expressive decrease in the maximum production time can be accomplished.



**Fig. 4.1.8:** Effect of phosphates concentration on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in wheat bran based-SSF and SmF media.

### Influence of NaCl on enzyme production in SSF and SmF

The influence of NaCl concentration on the production of  $\alpha$ -L-AFase when wheat bran was used as a carbon source in SSF and SmF media is presented in **Figure 4.1.9**. The maximal  $\alpha$ -L-AFase yield was obtained in SSF and SmF media with the concentration of 2.5 % of NaCl. The enzyme yield markedly decreased in SSF and SmF media with lower NaCl concentration. However, no significant effect was observed in enzyme production when higher concentrations of NaCl were used (Fig. 4.1.9). Although, the exact reason for increasing the  $\alpha$ -L-AFase production in SSF and SmF with the increase in NaCl concentration is not clear. However, it seems that NaCl enhance the stability of the enzyme against inactivation (Joshi *et al.*, 2006).

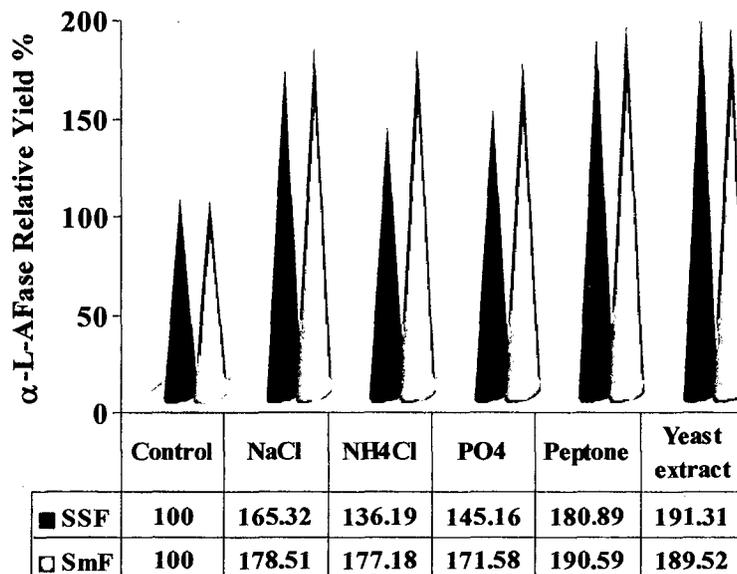


**Fig. 4.1.9:** Effect of NaCl concentration on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in wheat bran based-SSF and SmF media.

Although, higher NaCl concentration was reported in the growth medium of *Arthrobacter* sp that produce  $\alpha$ -L-AFase under SSF conditions (Khandeparkar *et al.*, 2008). Furthermore, it was reported that salt stress is an environmental signal affecting degradative enzymes synthesis in *Bacillus subtilis* (Kunst and Rapoport, 1995).

## Assessment of the culture response to some SSF and SmF media components

To evaluate the response of *Bacillus* sp NIOCCW19 to some SSF and SmF media components that has been studied, the enzyme production in the presence and absence of the optimum concentration of medium component was considered. The relative yields of  $\alpha$ -L-AFase was calculated as the change in enzyme production in the presence of optimum concentration of the component under study in medium relative to that obtained in the controlled medium free from that medium component. **Figure 4.1.10** shows the response of the culture to some SSF and SmF media components. The culture showed a wide range of response to the optimum concentrations of SSF and SmF media components. The response represented by the increase in  $\alpha$ -L-AFase yield which ranged from 136 % to 191 % and from 171% to 189 % in SSF and SmF, respectively.



**Fig. 4.1.10:** The response of the culture to some SSF and SmF media components. Control: 100 % is equivalent to enzyme yield (U/g IDS) obtained in the absence of the medium component. Relative yield calculated as the change in enzyme production in the presence of optimum concentration of the component under study in medium relative to that obtained in the controlled medium.

### Growth and enzyme production under SSF and SmF using agro-industrial residues

During the time course study,  $\alpha$ -L-AFase activity was detected in the culture supernatant from the first day till the sixth day and the major peak of activity was found on the fourth day in wheat bran, oat bran, sugar cane bagasse, sugar beet pulp and rice husks-based SSF as well as SmF media (Fig. 4.1.11a and Fig. 4.1.11b).

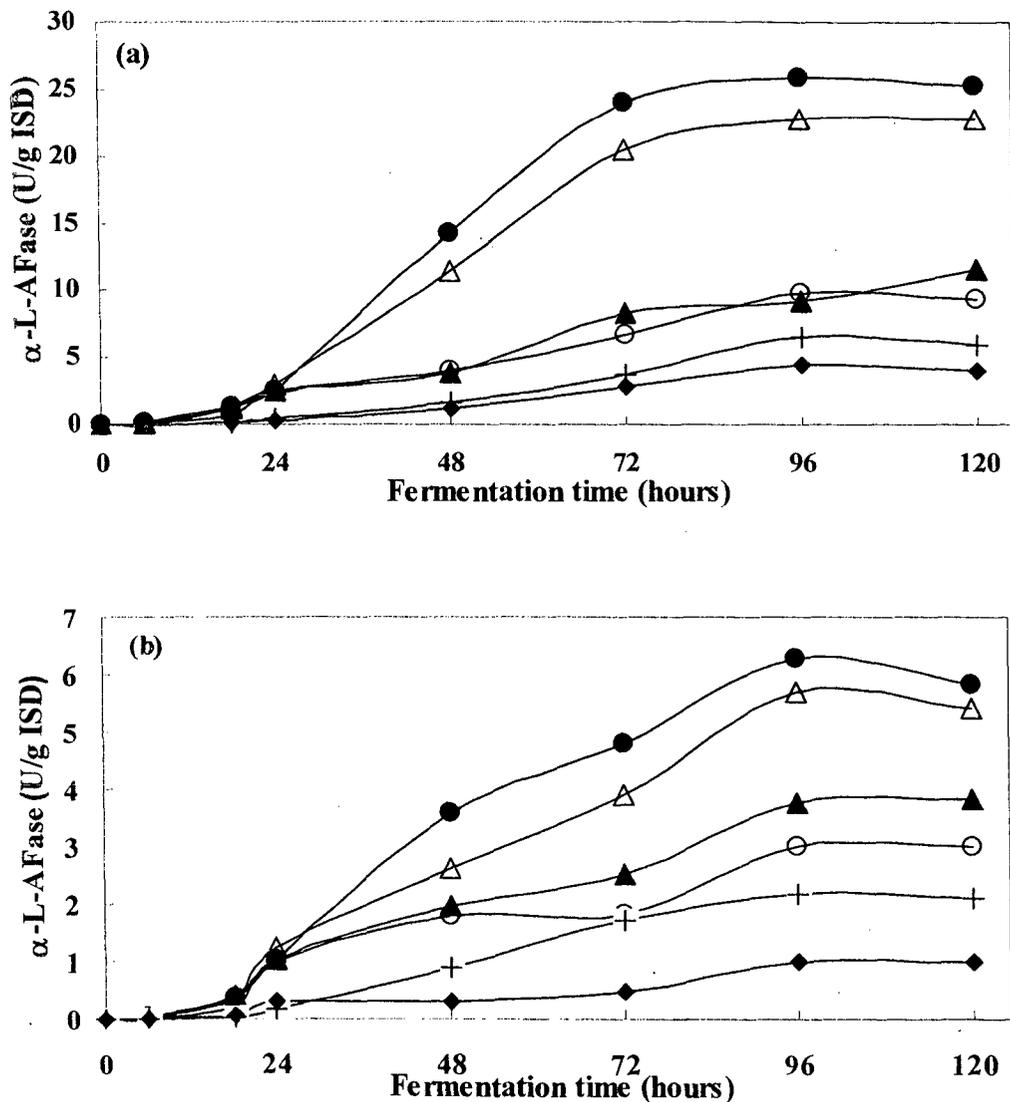
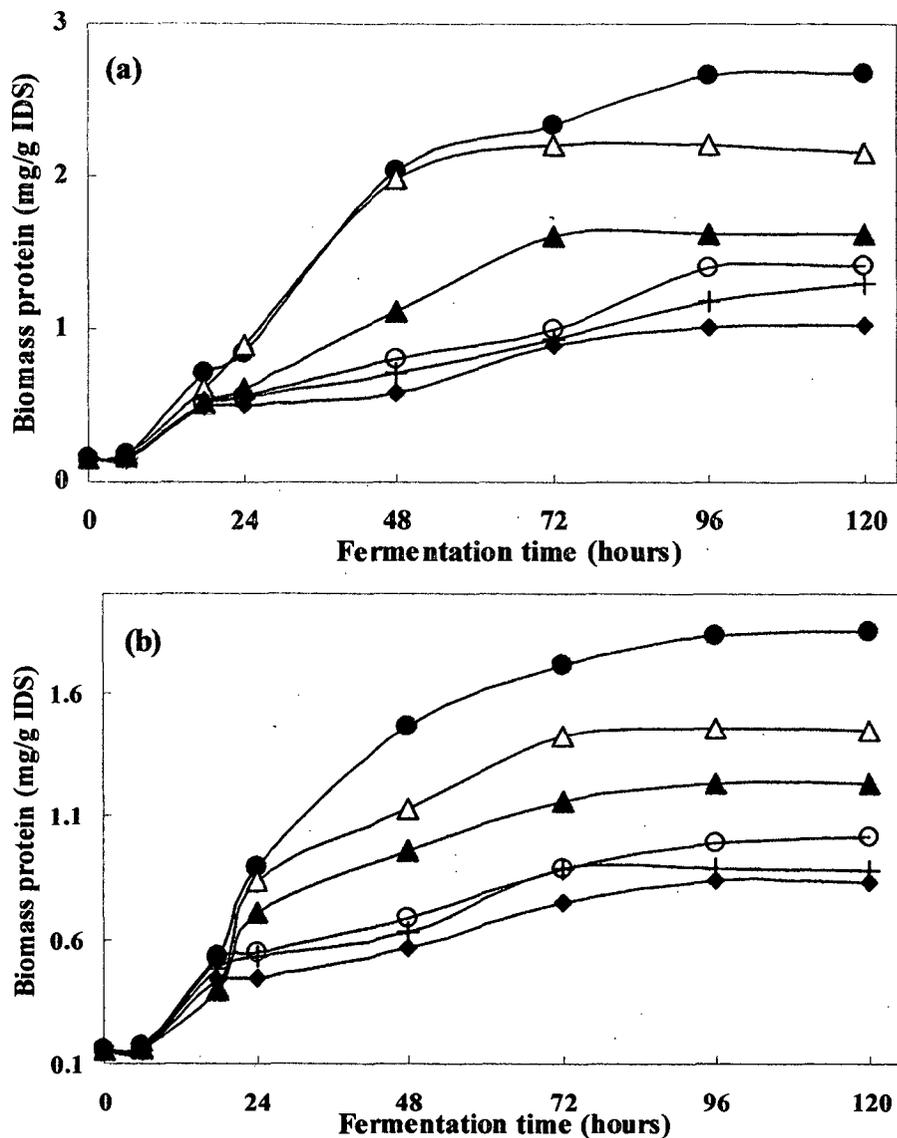


Fig. 4.1.11: Effect of incubation time on  $\alpha$ -L-AFase production by *Bacillus* sp. when grown under (a) solid state fermentation, (b) submerged cultivation on : (●) wheat bran, (Δ) oat bran, (▲) oat spelt xylan, (○) sugar cane bagasse, (+) sugar beet pulp, (◆) rice husks.  $\alpha$ -L-AFase activity was assayed from the cell free supernatant at 75°C.

However,  $\alpha$ -L-AFase major peak of activity was found on the fifth day in oat spelt xylan-based SSF and SmF media. When wheat bran and oat bran were used a slight reduction in enzyme yield was observed after the optimum incubation period. This was probably due to depletion of nutrients available to the bacterium. Similar findings have been reported by other workers also (Chu *et al.*, 1992; Gupta *et al.*, 2002). Optimization of incubation time is important to avoid the effect of the metabolic enzymes such as proteases and transglycosidases that may also affect the actual yield of extracellular enzymes (Hrmova *et al.*, 1991; Penbroke *et al.*, 1992; Kulkarni *et al.*, 1999). These enzymes are optimally expressed at the end of the exponential phase; and the harvesting time of the extracellular enzymes must be correlated to the production of these enzymes on the medium under consideration (Kulkarni *et al.*, 1999).

The time course of bacterial growth on various agro-industrial residues was studied simultaneously with  $\alpha$ -L-AFase production. The agro-industrial residues that produced maximum bacterial biomass in SSF and SmF were those which induced maximum  $\alpha$ -L-AFase production (**Fig. 4.1.12a** and **Fig. 4.1.12b**). Production of bacterial biomass was higher on wheat bran and oat bran, whereas it was lower on rice husks, sugar beet pulp when grown under both SSF and SmF conditions (**Fig. 4.1.12a** and **Fig. 4.1.12b**). An early stationary phase of bacterial growth was observed at 72h of cultivation on most of the substrates in SSF and SmF. However, this growth phase was observed later at 96h when wheat bran and sugar cane bagasse were used in SSF and SmF. Comparing the patterns of bacterial growth on different substrates with  $\alpha$ -L-AFase production suggested that enzyme production followed the bacterial

growth up to 96 h in both cultivation systems. It has been noticed that SSF resulted in high bacterial biomass compare to that obtained in SmF on all agro-industrial residues. This was not surprising as it has been reported that as compared to SmF, SSF usually produce higher microbial biomass (Lekha and Lonsane, 1994; Romero-Gomez *et al.*, 2000; Diaz-Godinez *et al.*, 2001; Aguilar *et al.*, 2001a; Asther *et al.*, 2002).



**Fig. 4.1.12:** Growth curves (biomass production vs. incubation time) of *Bacillus* sp. when grown under (a) solid state fermentation, (b) submerged cultivation on: (●) wheat bran, (Δ) oat bran, (▲) oat spelt xylan, (○) sugar cane bagasse, (+) sugar beet pulp, (◆) rice husks. Biomass measured as bacterial cell protein according to Kanderparker and Bhosle (2006).

### **Comparative evaluation of $\alpha$ -L-AFase yields by SSF and SmF**

Bacteria such as *Bacillus* sp occupy a habitat which is characterized by constant changes in water availability, salinity, temperature, pH and nutrients type and availability. Thus, they have to cope with adverse conditions caused by the changes in these variables. It was reported that for the survival of *Bacillus subtilis*, the bacterium possess an adaptation system allowing protection against adverse conditions in the environment (Boch *et al.*, 1994; Potts, 1994; Kunst *et al.*, 1995 ). There are several reports claiming that SSF cultivation of fungi results in greater enzymes yields than those obtained under SmF (Pandey *et al.*, 1999; Solis-Pereira *et al.*, 1993; Viniegra-Gonzalez *et al.*, 2003). However, few reports on enzymes production by bacterial cultivation in SSF and SmF described similar finding (Archana and Satyanarayana, 1997; Krishna, 1999; Kapoor *et al.*, 2002; Kashyap *et al.*, 2003). Furthermore, to the best of our knowledge, there is no study on the simultaneous production of  $\alpha$ -L-AFase by cultivated bacteria under SSF and SmF systems.

The data in **Table 4.1.3** were considered for a comparative evaluation of  $\alpha$ -L-AFase yields by *Bacillus* sp NIOCCW19 in SSF and SmF following Sandhya *et al.* (2005). The data presented in **Table 4.1.3** represents the maximum enzyme yields obtained by using the optimum variables conditions of wheat bran based-SSF and SmF media. **Tables 4.1.3** also shows the fold increase in  $\alpha$ -L-AFase yields which were accomplished in SSF compared to SmF in the conducted experiments. An average of 4.56 fold increase in the yield of  $\alpha$ -L-AFase was accomplished in SSF compared to SmF. The result showed that total  $\alpha$ -L-AFase yield from 1 g wheat bran based-SSF medium was even higher than that could be produced from 250 ml wheat bran based-SmF

medium. Similar observation was reported for bacterial cellulases (Krishna, 1999).

**Table 4.1.3:**  $\alpha$ -L-AFase production (U/g IDS) by *Bacillus* sp using optimum variables conditions in wheat bran based-SSF and SmF processes.

Process variable	SSF medium	SmF medium	$\alpha$ -L-AFase SSF	$\alpha$ -L-AFase SmF	Folds increase in enzyme yield
NaCl (%)	2.50	2.50	24.65	5.47	4.51
NH <sub>4</sub> Cl (mg/ml)	2.25	2.25	24.65	5.22	4.72
PO <sub>4</sub> (mg/ml)	0.50	0.50	25.33	5.91	4.29
Peptone (mg/ml)	0.50	1.00	25.30	5.52	4.58
Yeast extract (mg/ml)	2.00	4.00	26.94	6.06	4.45
pH	6-10	8.00	25.68	6.09	4.22
Inoculum (ml)	1.00	3.00	24.30	5.23	4.65
Moisture level (v:w)	3:1	-	25.43	-	-
Temperature (°C)	30-40	40	23.80	4.67	5.09
					<b>Ave. 4.56</b>

Comparative studies on fungal production of alkaline and neutral proteases in SSF and SmF showed that total proteases activity present in 1g bran (SSF) were equivalent to 100 ml and 150 ml broth (SmF), respectively (George *et al.*, 1997; Sandhya *et al.*, 2005).

The marked differences in  $\alpha$ -L-AFase yield in submerged and solid state fermentation could be attributed to various reasons such as (i) osmotic gradient due to heterogeneous distribution of solvents, (ii) the matrix structure due to substrate porosity and adsorption forces present in SSF (iii) low water activity (aw) of the solid substrate which has a significant effect on the physiological activity of microorganisms and enzyme production (Antier *et al.*, 1993; Kapoor *et al.*, 2002) and (iv) the solid substrate not only supplies the nutrient to

microbial cultures growing in it but also serves as anchorage for the cells (Archana and Satyanarayana, 1997; Pandey *et al.*, 2000) allowing them to utilize the substrate more effectively and produce high enzymatic yields (Kashyap *et al.*, 2003). Moreover, it was suggested that, in *B. subtilis* the regulatory genes affect differently the expression of target genes under different growth conditions (O'Reilly *et al.*, 1994). The close proximity of cells on surfaces such as in SSF causes physiological responses distinct from those of suspension cultures such as in SmF. Thus, many bacterial activities such as the production of extracellular enzymes under SSF and SmF may be influenced differently at a genomic level (O'Reilly *et al.*, 1994; Kunst *et al.*, 1994; Msadek *et al.*, 1995). Furthermore, the extracellular localization of secreted proteins differs greatly between liquid and solid cultures (Iwashita *et al.*, 1998; Hashimoto and Nakata, 2003). On a solid substrate, the growing bacteria are anchored to solid substrate. In this case the secreted enzymes most probably are immobilized in the substrate during SSF. Consequently, their hydrolytic activity is limited to substrate in the close vicinity of the attached bacteria (Trinci, 1974; Prosser, 1994; Dynesen and Nielsen, 2003). In liquid fermentation, the secreted proteins are released into the culture medium, resulting in substrate degradation in the whole broth. However, the intensity of substrate degradation in SmF could be less than that in SSF. This is because the immobilized enzymes in SSF are more concentrated and in the close vicinity of their substrate. In contrast, the secreted enzymes in SmF are more diluted and have less chance to get attached to their substrate. Thus, the immobilized enzymes in SSF are more effective in degradation of the substrate as compared to the secreted ones in SmF (Pandey *et al.*, 2000). The carbohydrate degradation products usually are

the main inducers for hydrolytic enzymes production in both SSF and SmF. The difference in the presence of these inducers may result in different induction capabilities which in turn results in different enzymes production capabilities in SSF and SmF. These inducers are continuously present in large amounts, and in close proximity to the bacterium in SSF as compared to SmF (Iwashita, 2002; Te Biesebeke *et al.*, 2002). Moreover, Te Biesebeke *et al.* (2005) proposed that the processes of carbohydrate breakdown and consumption differ in SmF and SSF cultivations, and play important role in enzyme induction. Consequently, the availability of carbohydrate degradation products will also differ in SmF and SSF cultivation. Moreover, there are differences in the environmental conditions for microbial growth in SSF and SmF. In contrast to submerged fermentation, SSF is characterized by gradients of nutrients, oxygen, temperature and water availability and, as a consequence, differences in transcriptional regulation are expected (Iwashita, 2002; Te Biesebeke *et al.*, 2002). The transcription of genes coded for hydrolytic enzymes is induced by the solid substrate and by the SSF-specific environmental conditions. The SSF-specific environmental conditions include the physical nature of substrate surface, high temperature, and low  $a_w$  (water activity) (Te Biesebeke *et al.*, 2002). Moreover, it was found that transcription of genes coded for glucoamylase A and glucoamylase B in *Aspergillus niger* grown under SmF and SSF is regulated by pH (Panelva and Arst, 2002; Te Biesebeke *et al.*, 2005). However, Ishida *et al.* (2000) suggested that the induction of genes transcription is due mainly to these SSF-specific environmental conditions (Ishida *et al.*, 2000).

It was significant to note that in SSF lower inoculum levels were sufficient to achieve optimum yield of  $\alpha$ -L-AFase. The production of bacterial cellulase (Krishna, 1999), pectinase (Kashyap *et al.*, 2003), polygalacturonase (Kapoor *et al.*, 2002) and fungal proteinase (Sandhya *et al.*, 2005) showed similar observations. According to Sandhya *et al.* (2005) this might be due to natural growth conditions available to the cells in solid cultures as well as due to nutrients availability in the SSF medium (Sandhya *et al.*, 2005). It was interesting to note that lower concentrations of peptone and yeast extract were sufficient to attain optimum  $\alpha$ -L-AFase yield in SSF compared to SmF. This phenomenon should be taken in consideration while formulating the medium for production of  $\alpha$ -L-AFase in SSF and SmF. The pH of growth medium has less effect on  $\alpha$ -L-AFase yields in SSF compared to SmF. Furthermore, a broad range of incubation temperature was suitable to accomplish optimum  $\alpha$ -L-AFase yields in SSF and SmF.

Results obtained in this study indicated that, among the various agricultural residues studied, wheat bran was the most suitable agro-industrial residue for  $\alpha$ -L-AFase production by *Bacillus* sp NIOCCW19 in SSF as well as SmF. SSF proved its superiority in production of  $\alpha$ -L-AFase with less requirements of nitrogen source and under wide ranges of pH and temperature. Moreover, as wheat bran is a cheap and readily available agro-industrial byproduct, the production of  $\alpha$ -L-AFase using wheat bran based-SSF may be a cost-effective process. The study also revealed the possibilities of effective utilization of not only wheat bran but also other agricultural residues for enzymes production through biotechnological means. Such processes would not only help in reducing the cost of enzymes production but also participate in effective solid

waste management. We suggest that, the production of  $\alpha$ -L-AFase by *Bacillus* sp NIOCC W19 is controlled in response to the environment factors, nutritional status and culture conditions specific to the system. Furthermore, present data may be useful in the process scale up for industrial production of  $\alpha$ -L-AFase under SSF. This is because of high yield and the advantage of using concentrated fermentation broth in SSF system with a lower risk of contamination and a lower recovery cost related to higher enzyme yields as compared to SmF. As far as we are concerned, this work is the first contribution towards understanding the effect of some processes variables on the production of  $\alpha$ -L-AFase by a bacterium grown under SSF and SmF.

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Isolation, Purification, Characterization and Substrate Specificity of  
 $\alpha$ -L-Arabinofuranosidase Produced by *Bacillus* sp NIOCCW19  
Grown under Solid-State Fermentation

#### 4.2.1 INTRODUCTION

Solid state fermentation (SSF) is a well known cultivation system for fungi and few bacteria (Selvakumar and Pandey, 1999; Pandey *et al.*, 2000; Pandey, 2003). SSF has been investigated for production of some enzymes from some bacteria (Satyanarayana, 1994; Babu and Satyanarayana, 1995; Archana and Satyanarayana; 1997; Gessesse and Mamo, 1999; Selvakumar and Pandey, 1999; Virupakshi *et al.*, 2005). However, to the best of our knowledge there is only one report on the production of bacterial  $\alpha$ -L-arabinofuranosidase ( $\alpha$ -L-AFase) using SSF (Khandeparkar *et al.*, 2008). Yet, detailed study on the properties of bacterial  $\alpha$ -L-AFase produced under SSF is not reported. Some of these properties include substrate specificity and the role of the enzyme in hydrolysis of hemicelluloses. In fact, such investigation is of importance for many exciting industrial application such as bioethanol production from lignocelluloses. Furthermore, it has been found that *Bacillus* sp NIOCCW19 produce higher amount of  $\alpha$ -L-AFase when grown under SSF compared to submerged fermentation (SmF) (Section 1 of Chapter 4). Considering the economic feasibility of using rather inexpensive easily available raw substrates, the advantages of SSF, and to contribute toward understanding the properties of bacterial  $\alpha$ -L-AFase produced under SSF, it was essential to study the  $\alpha$ -L-AFase produced by *Bacillus* sp NIOCCW19 when grown under SSF.

Therefore, this section presents studies on isolation, purification, characterization, and substrate specificity of  $\alpha$ -L-arabinofuranosidase produced by *Bacillus* sp NIOCCW19 grown on wheat bran under solid-state fermentation.

## **4.2.2 MATERIALS and METHODS**

### **4.2.2.1 Culture and growth conditions**

*Bacillus* sp NIOCC-W19 was grown and maintained as described in Section 1 of Chapter 4.

### **4.2.2.2 Enzyme Assay**

$\alpha$ -L-AFase activity estimation was conducted as described in Section 1 of Chapter 4.

### **4.2.2.3 Mass production and isolation of $\alpha$ -L-AFase**

Mass production of  $\alpha$ -L-AFase by *Bacillus* sp was conducted under solid state fermentation. Erlenmeyer flasks (500 ml) containing wheat bran (10g) moisturized with SSF-MBSM2 in ratio of (1: 3, w:v) was used to grow the bacterial cells at 40 °C. The composition of SSF-MBSM2 is given in **Table 4.1.1** (Section 1, Chapter 4). The fermented medium of the grown culture was harvested in stationary growth phase. It was mixed thoroughly with Na-phosphate buffer (50 mM, pH 8.0) on orbital shaker 200 RPM for 1 h and centrifuged (10,000 RPM) at 4°C for 10 min. The supernatant was concentrated using Amicon ultrafiltration stirred cell (Amicon, USA) as described in Chapter 3. Solid ammonium sulfate was slowly added with stirring to the ultrafiltered supernatant to a 55% saturation level at 4°C. The mixture was left for another 60 min, and the resulting precipitate was removed by centrifugation at 10,000 RPM at 4°C for 10 min. Dialysis was conducted as described in Chapter 3. The dialysate was concentrated using rotary vacuum evaporator at 40°C.

#### **4.2.2.4 Purification of $\alpha$ -L-AFase**

Purification of  $\alpha$ -L-AFase produced by *Bacillus* sp was performed by using gel filtration and ion exchange chromatography as described in chapter 3.

#### **4.2.2.5a Evaluation of purity and molecular mass determination**

SDS-PAGE electrophoresis was performed as described in chapter 3. The standard molecular weight markers ranging from 24 to 205 kDa (Sigma) were used.

#### **4.2.2.5b Native PAGE and zymogram analysis**

Native PAGE and zymogram analysis were performed as described in chapter 3. The native gel was sandwiched to the detection gel, which contained 1% agarose and 1mM p-nitrophenyl- $\alpha$ -L-arabinofuranoside (Sigma) dissolved in 50 mM Na-phosphate buffer (pH 7.0).

#### **4.2.2.6 Effect of temperature on $\alpha$ -L-AFase activity and stability**

Effect of temperature on the activity and stability of purified  $\alpha$ -L-AFase (0.5 U) were assessed at temperatures range from 30°C to 90°C. The procedures followed were described in chapter 3, and the standard enzyme assay described Section 1 of Chapter 4.

#### **4.2.2.7 Effect of pH on $\alpha$ -L-AFase activity and stability**

Effect of pH on the activity and stability of purified  $\alpha$ -L-AFase (0.5U) was assessed in pH range from pH 3 to pH 12. The procedures followed were described in chapter 3 and the standard enzyme assay described Section 1 of Chapter 4.

#### **4.2.2.8 Kinetics studies of $\alpha$ -L-AFase**

The kinetic constants  $K_m$  and  $V_{max}$  were determined as described in Chapter 3 using *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside in 50mM Na-phosphate buffer, pH 8 at 75 °C.

#### **4.2.2.9 Effect of Metal ions and other chemical agents on $\alpha$ -L-AFase**

The purified enzyme was incubated in 1 mM solution of either  $Ca^{2+}$ ,  $Cu^{2+}$ ,  $Co^{2+}$ ,  $Fe^{2+}$ ,  $Hg^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ , DTT, SDS, EDTA and L-arabinose (100mM) for 1 h at room temperature ( $28 \pm 2^\circ C$ ). Residual activity was measured using standard enzyme assay described in Section 1 of Chapter 4.

#### **4.2.2.10 Amino acid composition of $\alpha$ -L-AFase**

Amino acids composition of the enzyme was determined using HPLC as described in chapter 3.

#### **4.2.2.11 Determination of Substrates Specificity of $\alpha$ -L-AFase**

For determination of  $\alpha$ -L-AFase substrates specificity, the enzyme was reacted with *p*-nitrophenyl-aryl glycoside synthetic substrates,  $\alpha$ -L-arabinofuranooligosaccharides and other arabinose containing substrates as described in Chapter 3. TLC was used for analysis of the reactions products as described in Chapter 3. Substrate preparation, reactions conditions and TLC analysis were as these described in chapter 3.

#### **4.2.2.12 Determination of hydrolysis rates of hemicellulosic substrates**

Hydrolysis rates of some hemicellulosic substrates were estimated following the methods described in chapter 3. The hemicellulosic substrates used were wheat  $\alpha$ -D-arabinoxylan, arabinogalactan, arabinan, debranched arabinan and oat spelt xylan. The  $\alpha$ -L-AFase was reacted with either of the above hemicellulosic substrates and the reaction mixture contained 0.5 ml of  $\alpha$ -L-AFase solution (0.5 U) and 1mg of substrate in 0.1 ml of 50 mM sodium phosphate buffer

(pH 8.0). After 48 h incubation at 50°C, an aliquot of each reaction mixture was taken and the reducing sugar formed from each substrate was measured by Somogyi-Nelson Method (1952).

Each experimental value in this section represented the average of two independent determinations. The standard deviations (SD±) of these readings were equal or less than 6.3 % of the calculated means.

#### 4.2.3 RESULTS AND DISCUSSION

##### Isolation and purification of $\alpha$ -L-AFase

The enzyme isolation and purification were performed at room temperature ( $28 \pm 2$  °C). The  $\alpha$ -L-AFase was purified to homogeneity from culture filtrate of *Bacillus* sp grown on wheat bran under SSF. **Table 4.2.1** summarizes the procedures for the purification of extracellular  $\alpha$ -L-AFase from *Bacillus* sp.

**Table 4.2.1:** Purification of  $\alpha$ -L-AFase enzyme isolated from *Bacillus* sp.

Steps	Enzyme activity U	Protein mg	Specific activity U/mg	Purification fold	Yield %
Supernatant	896.12	1148.87	0.78	1.00	100.00
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	623.90	311.95	2.00	2.56	69.62
Sephadex G-200	349.50	19.58	17.85	22.89	39.00
DEAE Sepharose <sup>FF</sup>	273.26	2.98	91.70	117.56	30.49
CM Sepharose <sup>FF</sup>	90.15	0.28	323	414.10	10.06

The crude extract of the enzyme was concentrated by ultrafiltration with Amicon ultrafiltration stirred cell (Amicon, USA) equipped with membrane filter (Millipore, Bedford, MA, USA) with 30 kDa MW cut-off.  $\alpha$ -L-AFase activity was found only in the retentate. Ammonium sulfate precipitation of the enzyme increased the purification fold to 2.56. Gel filtration using Sephadex

G-200 size exclusion column chromatography resulted in a multiple peaks of the eluted proteins (Fig. 4.2.1a). When these were screened one peak of the  $\alpha$ -L-AFase activity was found (Fig. 4.2.1a). The fractions that showed  $\alpha$ -L-AFase were collected. When the enzyme protein was further loaded onto anion exchange DEAE Sepharose FF column chromatography, it resolved into two protein peaks, one major and another minor peak. The major peak showed  $\alpha$ -L-AFase activity (Fig. 4.2.1b).

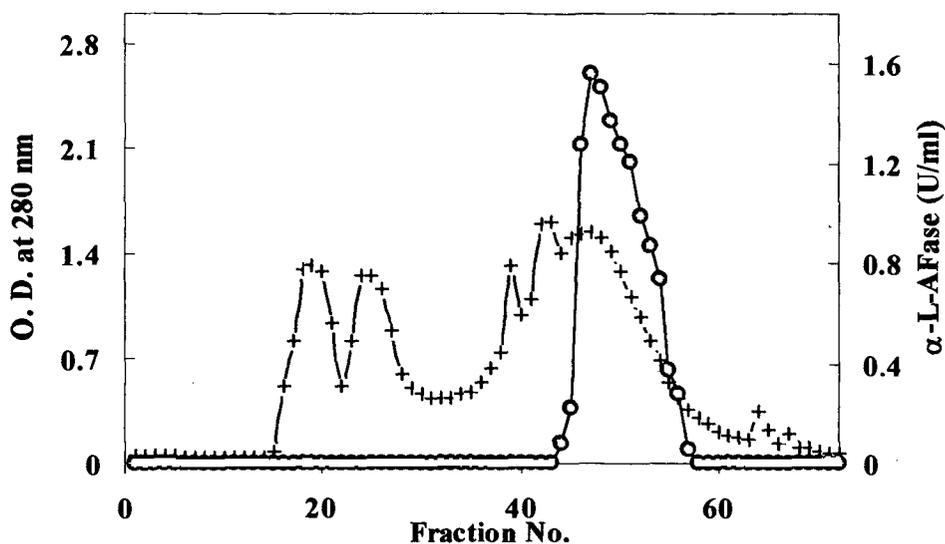


Fig. 4.2.1a: Elution profile of *Bacillus* sp.  $\alpha$ -L-AFase and xylanase on Sephadex G-200. (+) Protein profile at 280 nm (o) Fractions containing  $\alpha$ -L-AFase activity.

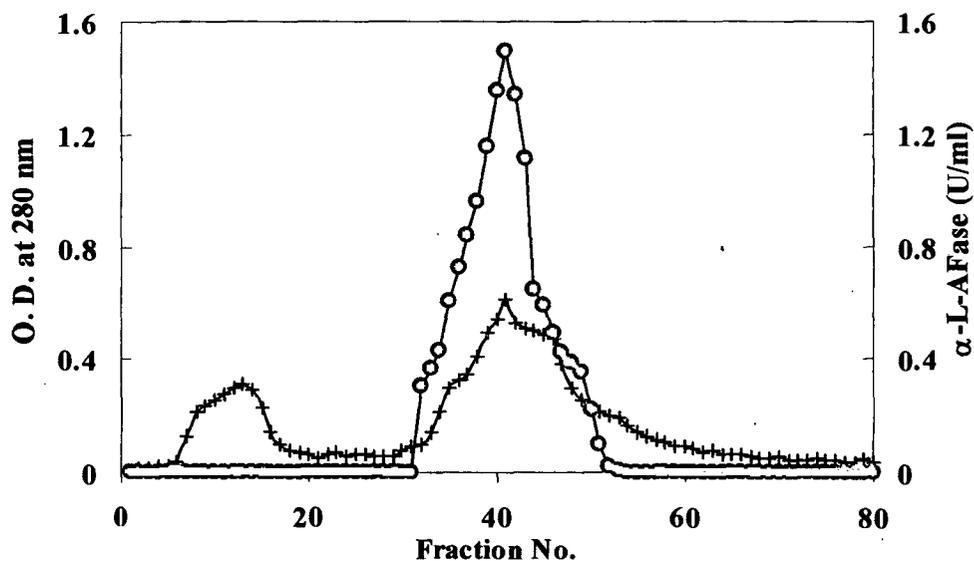
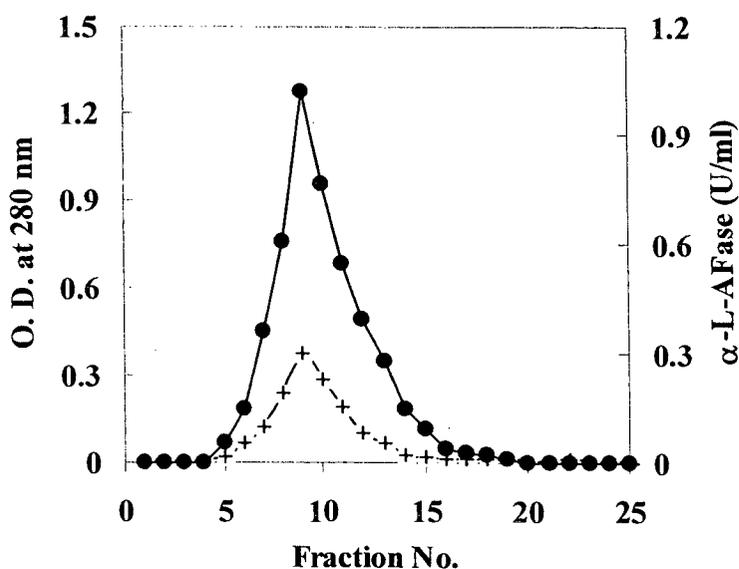


Fig. 4.2.1b: Elution profile of *Bacillus* sp.  $\alpha$ -L-AFase on DEAE-Sepharose FF. (+) Protein profile at 280 nm (o) Fractions containing  $\alpha$ -L-AFase activity.

The major peak protein fraction with  $\alpha$ -L-AFase activity was further loaded onto cation exchange CM-Sepharose FF column. When eluted with linear NaCl gradient up to 1.5 M; a single protein peak with  $\alpha$ -L-AFase activity was obtained (Fig. 4.2.1c). The overall level of recovery was 10% while 414-fold purification of  $\alpha$ -L-AFase was achieved with a specific activity of 323.1 U/mg (Table 4.2.1). After following these purification steps, the enzyme preparation from *Bacillus* sp showed only one form of  $\alpha$ -L-AFase.

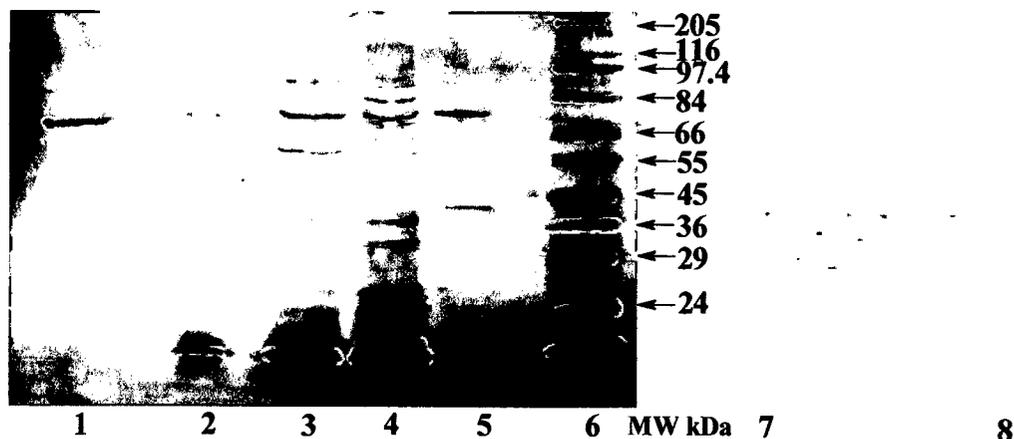


**Fig. 4.2.1c:** Elution profile of *Bacillus* sp.  $\alpha$ -L-AFase on CM-Sepharose FF. (+) Protein profile at 280 nm (•) Fractions containing  $\alpha$ -L-AFase activity.

### Molecular weight determination and Zymogram analysis

The molecular weight of the purified  $\alpha$ -L-AFase estimated from the relative mobility of the standard proteins on SDS-PAGE and native-PAGE. When applied on to SDS-PAGE polyacrylamide gels, preparation of the pure denatured enzyme yielded a single band of protein after staining with Coomassie brilliant blue (Plate 4.2.1, Lane-6). The MW was calculated to be ~75 kDa. Moreover, nondenatured enzyme preparation applied on native-PAGE electrophoresis resulted in one band of enzyme activity as

detected by pNPAF-agarose gel (Plate 4.2.1, Lanes-7 and 8). The results of SDS-PAGE and native-PAGE electrophoresis further confirmed the presence of single form of  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19.



**Plate 4.2.1:** SDS-PAGE and Zymogram analysis of purified  $\alpha$ -L-AFase from *Bacillus* sp. Lane1; the purified enzyme after CM-Sepharose FF; Lane2; Supernatant; Lane3; after Sephadex G200; Lane4; after  $(\text{NH}_4)_2\text{SO}_4$  precipitate; Lane5; after DEAE-Sepharose FF; Lane6; Molecular markers; Lane7; Zymogram of the enzyme from the crude extract, Lane8; Zymogram of pure enzyme.

In contrast, multiple forms of  $\alpha$ -L-AFase have been reported from fungi (Laborda *et al.*, 1973; Rombouts *et al.*, 1988; Ramon *et al.*, 1993; Luonteri *et al.*, 1995; Filho *et al.*, 1996; Kaneko *et al.*, 1998a; Koseki *et al.*, 2003) and actinomycetes (Tajana *et al.*, 1992). The  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 seems to be monomeric enzyme as only one protein band was detected on both native and denaturated electrophoresis gels. However, multimeric  $\alpha$ -L-AFases with high molecular weight reported from many bacteria related to *Bacillus* spp (Bezalel *et al.*, 1993; Gilead and Shoham, 1995; Morales *et al.*, 1995; Degrassi *et al.*, 2003; Kim *et al.*, 2004; Canakci *et al.*, 2007) (Table 4.2.2). On the other hand, monomeric  $\alpha$ -L-AFase with lower MW were found in many bacteria (Weinstein and Albersheim, 1979; Lee and Forsberg, 1987; Kaji and Shimikawa, 1984; Kaneko *et al.*, 1994; Debeche *et al.*,

**Table 4.2.2:** Biochemical characteristics and substrates specificity of  $\alpha$ -L-AFase isolated from *Bacillus* sp NIOCCW19 and its comparison with other  $\alpha$ -L-AFases from the genus *Bacillus* and its related bacteria.

Bacteria	Mw kDa	Opt. pH	Opt. Temp.°C	$K_m$ mM	$V_{max}$ U/mg	Active on Substrates	References
<i>subtilis</i> .	65	6.5	ND	ND	ND	AOS,BA, , not active on AX	Weinstein and Albersheim, (1979)
<i>Stearothermophilus L1</i>	110	7	70	0.22	110	AX,AG	Bezalel <i>et al.</i> , (1993)
<i>subtilis 3-6</i>	61	7	60	ND	ND	AOS, BA, not active on AX, AG	Kaneko <i>et al.</i> , (1994)
<i>polymyxa</i>	166	6.5	50	0.324	214.1	AOS, not active DA, AX, AG	Morales <i>et al.</i> , (1995)
<i>stearothermophilus T-6</i>	256	5.5-6	70	0.42	749	BA , low activity on OSX	Gilead and Shoham, (1995)
<i>ermobacillus xylanilyticus</i>	56.071	5.6 - 6.2	75	0.5	555	AX,OSX	Debeche <i>et al.</i> , (2000)
<i>pumilus</i>	220	7	55	1.3	52.9	ND	Degrassi <i>et al.</i> , (2003)
<i>stearothermophilus</i> No. 236	190	6.5	55	1.19	26.1	AX, not active on OXS	Kim <i>et al.</i> , (2004)
<i>obacillus caldoxylolyticus TK4</i>	236	6.0	75-80	0.17	588.2	AOS,BA, not active on AX,AG	Canakci <i>et al.</i> , (2007)
<i>cillus sp.NIOCCW19</i>	~75	8	75	0.46	323.1	AOS, BA, DA, AG, AX, OSX	Present study

OS: arabinofuranooligosaccharides, AX: arabinoxylan, BA: branched arabinan, AG: arabinogalactan DA: debranched arabinan, OSX: oat speltan, ND: not determined.

2000; Mai *et al.*, 2000; Beylot *et al.*, 2001a) and actinomycetes (Kaji *et al.*, 1981; Komae *et al.*, 1982; Tajana *et al.*, 1992; Manin *et al.*, 1994; Matuso *et al.*, 2000; Tsujibo *et al.*, 2002; Tuncer and Ball, 2003a).

### Effect of temperature on the activity and stability of $\alpha$ -L-AFase

The  $\alpha$ -L-AFase from *Bacillus* sp showed a very low activity at room temperature. A gradual increase in the enzyme activity was observed with the increase in temperature. The optimum activity of the enzyme was found to be at 75°C. When temperature increased over 75°C, the enzyme activity was decreased (Fig. 4.2.2). However, the decreased in enzyme activity was found to be more noticeable above 90°C which reached 52.7% of the optimum activity at this temperature (Fig. 4.2.2). The enzyme showed complete stability at 50°C for more than 24h. It retained 92.12% and 96.8 % of it original activity at 60°C and 70°C for 14 h and 7 h respectively. Moreover, the enzyme showed a half-life of about 7h, 4h and 20 min at 75°C, 80°C and 90°C, respectively (Fig 4.2.3a and Fig 4.2.3b). With respect to enzyme thermoactivity and thermostability, a direct comparison of the temperature optima and stability for  $\alpha$ -L-AFase activity among various microorganisms is difficult.

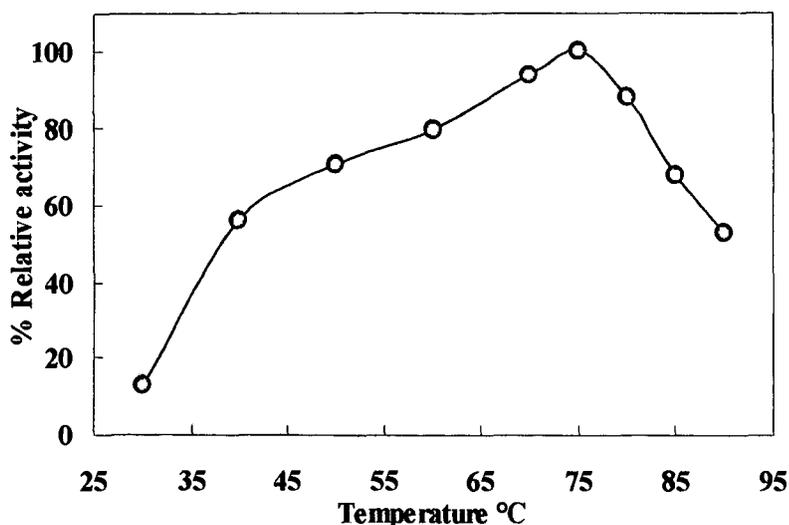
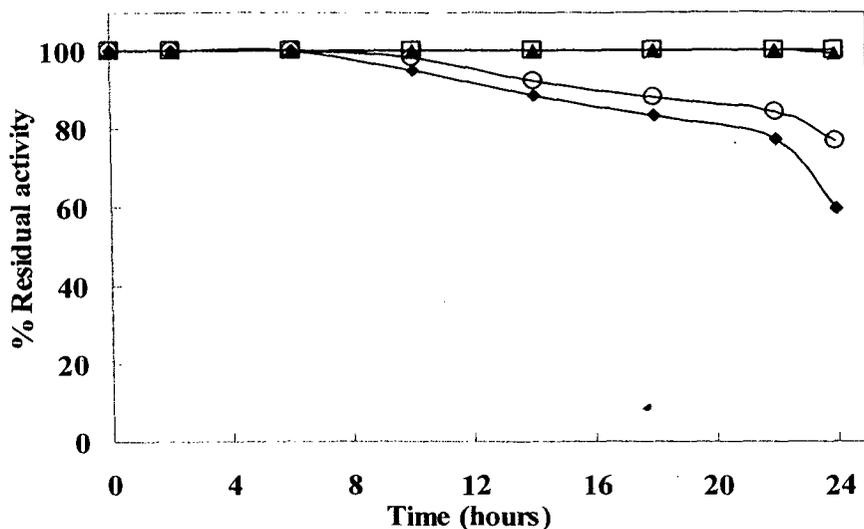
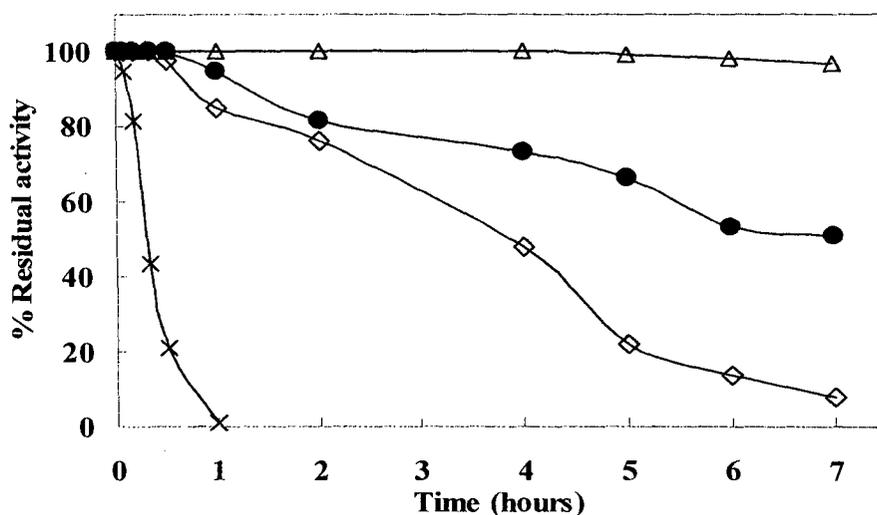


Fig. 4.2.2: Effect of temperature on the activity of  $\alpha$ -L-AFase of *Bacillus* sp.



**Fig.4.2.3a:** Thermal stability of *Bacillus* sp.  $\alpha$ -L-AFase. The pure enzyme was incubated in 50mM Na-phosphate buffer, pH 8.0 at 40°C (□), 50°C (▲), 60°C (○) and 65°C (◆) for different intervals and residual activity was determined at 75°C and pH 8.0.



**Fig.4.2.3b:** Thermal stability of *Bacillus* sp.  $\alpha$ -L-AFase. The pure enzyme was incubated in 50mM Na-phosphate buffer, pH 8.0 at 70°C (Δ), 75°C (●), 80°C (◇) and 90°C (×) for different intervals and residual activity was determined at 75°C and pH 8.0.

This is due to differences in the assay procedures and purity of enzymes used. However, based on the published literature several bacterial  $\alpha$ -L-AFases were reported with lower temperature optima and less thermostability than that found in  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19. Some of these are the  $\alpha$ -L-AFase

produced by *Pseudomonas* sp NIOCCAr27 (**Chapter 3**) and many other bacteria (Weinstein and Albersheim, 1979; Greve *et al.*, 1984; Lee and Forsberg, 1987; Kaneko *et al.*, 1994; Schyns *et al.*, 1994; Morales *et al.*, 1995; Schwarz *et al.*, 1995; Van Laere *et al.*, 1997; Renner and Breznak, 1998; Beylot *et al.*, 2001a; Kosugi *et al.*, 2002; Degrassi *et al.*, 2003; Margolles and de los Reyes-Gavilán, 2003; Birgisson *et al.*, 2004) and actinomycetes (kaji *et al.*, 1981; Komae *et al.*, 1982; Tajana *et al.*, 1992; Manin *et al.*, 1994; Vincent *et al.*, 1997; Matuso *et al.*, 2000; Tsujibo *et al.*, 2002; Mai *et al.*, 2000; Tsujibo *et al.*, 2002; Tuncer and Ball, 2003a). On the other hand,  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 found to be fairly superior with respect to temperature optima and stability than many  $\alpha$ -L-AFase reported from other *Bacillus* species (Weinstein and Albersheim 1979; Kaneko *et al.*, 1994; Morales *et al.*, 1995; Degrassi *et al.*, 2003; Kim *et al.*, 2004) (**Table 4.2.2**). The temperature optima of  $\alpha$ -L-AFase from *Bacillus* sp NIOCC-W19 was higher than that for  $\alpha$ -L-AFase from *B. stearothermophilus* T-6 (Gilead and Shoham, 1995), *B. stearothermophilus* (Bezalel *et al.*, 1993) and similar to that of  $\alpha$ -L-AFases from *Thermobacillus xylanilyticus* (Debeche *et al.*, 2000) and *Geobacillus caldoxylolyticus* TK4 (Canakci *et al.*, 2007) (**Table 4.2.2**). Furthermore, a study by Bergquist *et al.* (1999) showed that, the diversity between closely related bacteria suggests lateral transfer of blocks of genes between these bacteria in the past. As thermostable  $\alpha$ -L-AFases were also reported from *Thermobacillus xylanilyticus* (Debeche *et al.*, 2000) and *Geobacillus caldoxylolyticus* TK4 (Canakci *et al.*, 2007), this may suggest that a lateral genes transfer could be responsible for thermostability of  $\alpha$ -L AFase from *Bacillus* sp NIOCCW19. Furthermore, many reports suggest that the enzymes produced under solid state

fermentation are more thermostable than the once produced under submerged fermentation (Solis-Pereyra *et al.*, 1993; Raimbault, 1998; Pandey and Selvakumar, 1999; Kashyap *et al.*, 2003; Pandey, 2003; Couto and Sanromán; 2006). The  $\alpha$ -L-AFase with highest temperature optima and stability has been obtained from the hyperthermophilic *Thermotoga maritima* MSB8 (Miyazaki, 2005). The high thermostability of this enzyme has been attributed to the hyperthermophilic nature of the bacteria producing this enzyme. Furthermore, not many enzymes are stable at their temperature optima for long period of time. However,  $\alpha$ -L-AFases from *Rhodothermus marinus* (Gomes *et al.*, 2000) and *Aureobasidium pullulans* (Saha and Bothast, 1998) were stable at their optimal temperatures (75°C and 85°C) for more than 30 min. These enzymes also showed a half-life of about 8 and 8.3 h, respectively at their optimum temperatures. In contrast,  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 was also fairly stable at its optimum temperature (75°C) for about 50 min and has a half-life of about 7 h at same temperature. Moreover, most fungal  $\alpha$ -L-AFases were reported with lower temperature optima and less stability than that for  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19. In fact the temperature optima of  $\alpha$ -L-AFases from fungi were found to be between 50°C and 65°C (Rombouts *et al.*, 1988; Gunata *et al.*, 1990; Kaneko *et al.*, 1993; Ramon *et al.*, 1993; Fernandez-Espinar *et al.*, 1994; Kimura *et al.*, 1995; Wood and McCrae, 1996; De Ioannes *et al.*, 2000; Hashimoto and Nakata, 2003; Koseki *et al.*, 2003; Sakamoto and Kawasaki, 2003; Chacoń -Marténez *et al.*, 2004; Matsumura *et al.*, 2004).

### Effect of pH on the activity and stability of $\alpha$ -L-AFase

The  $\alpha$ -L AFase from *Bacillus* sp NIOCCW19 showed pH optima at 8.0. It also showed more than 50% relative activity at a broad range of pH ranging from pH 4 to pH 9 (Fig. 4.2.4). Similar pH activity range of  $\alpha$ -L AFases was not reported from any other *Bacillus* related species. With respect to pH stability, the enzyme showed almost complete stability at pHs from pH 6.0 to pH 9.0 for more than 24 h. It also retained 84.99%, 54.39% and 76.04% of its original activity after 24 h incubation at pH 10, pH 11 and pH 5.0 respectively (Fig. 4.2.5a and Fig. 4.2.5b). However, the enzyme was less stable at extreme acidic or basic pHs. In contrast,  $\alpha$ -L AFase from *Pseudomonas* sp NIOCCAr27 (Chapter 3) has pH optima at 7.0 and was stable for 4 h at pH from 5.0 to 9.0. On the hand, pH optima and stability of all the reported  $\alpha$ -L AFases from different strains of *Bacillus* spp were found to be between pH 5.5 and pH 7.0 (Weinstein and Albersheim, 1979; Bezalel *et al.*, 1993; Kaneko *et al.*, 1994; Gilead and Shoham, 1995; Morales *et al.*, 1995; Degrassi *et al.*, 2003; Kim *et al.*, 2004; Canakci *et al.*, 2007) (Table 4.2.2). Moreover,  $\alpha$ -L AFase from *Thermobacillus*

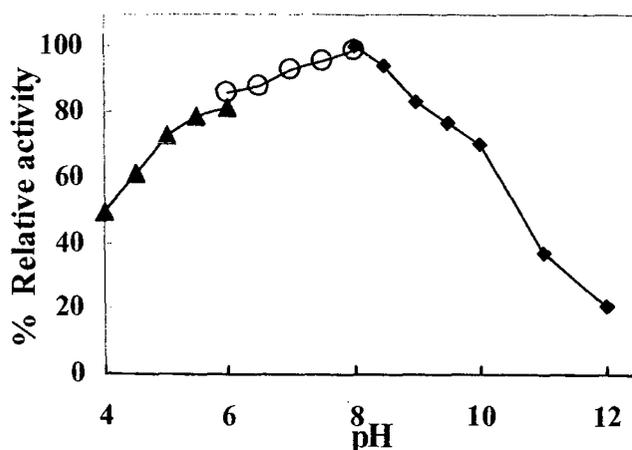
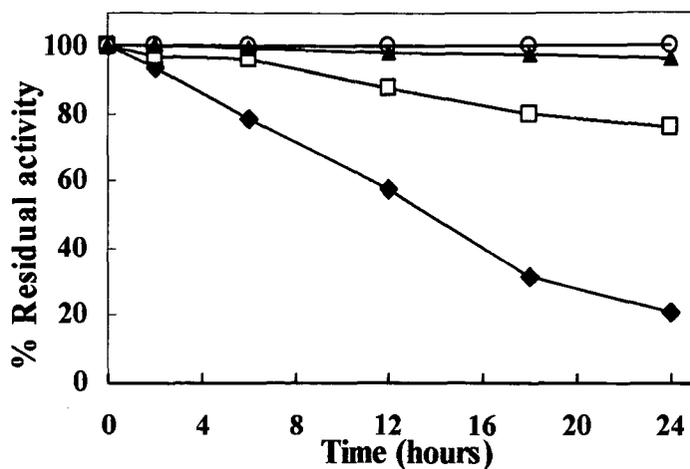
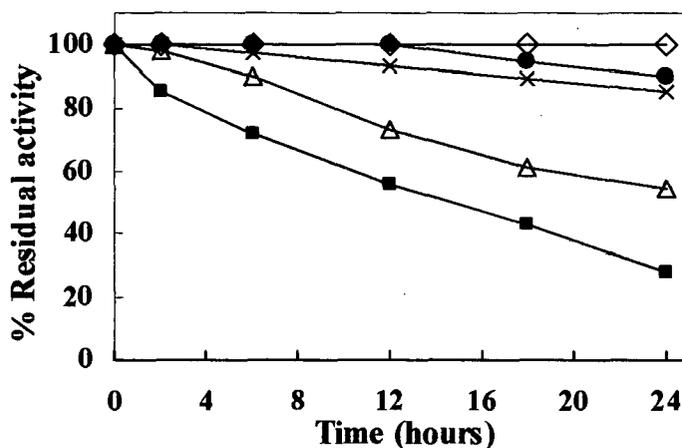


Fig. 4.2.4: pH profile *Bacillus* sp.  $\alpha$ -L-AFase. Enzyme activity was measured at 75°C using different buffers. The buffers used were: (▲) Citrate; (○) Phosphate; (■) Glycine- NaOH.



**Fig. 4.2.5a:** Effect of pH on stability of *Bacillus* sp.  $\alpha$ -L-AFase. The pure enzyme was incubated at room temperature up to 24 h in 50mM buffers. Buffers used were Citrate with pH 4 (♦) and pH 5 (□); Phosphate with pH 6 (▲) and pH 7 (○). Residual activity was assayed at pH 8.0 and 75°C.



**Fig. 4.2.5b:** Effect of pH on stability of *Bacillus* sp.  $\alpha$ -L-AFase. The pure enzyme was incubated at room temperature up to 24 h in 50mM buffers. Buffers used were Phosphate with pH 8 (◇); Glycine-NaOH with pH 9 (●); pH 10 (×), pH 11 (Δ), pH 12 (■). Residual activity was assayed at pH 8.0 and 75°C.

*xylanilyticus* (Debeche *et al.*, 2000) had pH optima between pH 5.6 and pH 6.2. According to Debeche *et al.* (2000) this enzyme was stable in prolonged incubation at a broad pH range between pH 4.0 and pH 12 (Table 4.2.2). The crude  $\alpha$ -L AFase from *R. marinus* was stable for 24 h at pH range from pH 5.0 to pH 9.0 (Gomes *et al.*, 2000). Furthermore, several other bacteria and actinomycetes were found to produce  $\alpha$ -L AFase with pH optima and stability

at pH range from pH 5.0 to pH 7.0 (Kaji *et al.*, 1981; Komae *et al.*, 1982; Greve *et al.*, 1984; Lee and Forsberg, 1987; Tajana *et al.*, 1992; Manin *et al.*, 1994; Schyns *et al.*, 1994; Schwarz *et al.*, 1995; Van Laere *et al.*, 1997; Vincent *et al.*, 1997; Renner and Breznak, 1998; Matuso *et al.*, 2000; Beylot *et al.*, 2001a; Kosugi *et al.*, 2002; Tsujibo *et al.*, 2002; Margolles and de los Reyes-Gavilán 2003; Birgisson *et al.*, 2004; Miyazaki, 2005). In contrast, all reported fungal  $\alpha$ -L-AFases were active and stable at acidic pH range from 3.0 to 6.0 (De Ioannes *et al.*, 2000; Hashimoto and Nakata, 2003; Koseki *et al.*, 2003; Sakamoto and Kawasaki, 2003; Matsumura *et al.*, 2004; Chacón-Martènez *et al.*, 2004). The pH activity and stability profiles of  $\alpha$ -L AFase from *Bacillus* sp NIOCCW19 suggested that the enzyme has potentials to work at broad pH range required for different industrial applications.

#### 4.2.3.5 Kinetic parameters of $\alpha$ -L AFase

The rate of hydrolysis with respect to p-nitrophenyl- $\alpha$ -L- arabinofuranoside was examined at pH 8.0 and 75°C. Substrate inhibition was not observed with pNPAF tested up to a 8 mM concentration (Fig. 4.2.6a). The  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 displayed typical Michaelis-Mentien kinetics, yielding a  $K_m$  of 0.46 mM and  $V_{max}$  of 323 U mg<sup>-1</sup> of protein as determined by a Lineweaver- Burke plot (Fig. 4.2.6b). The  $V_{max}$  of  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 was found to be higher than that reported for  $\alpha$ -L-AFase from *Pseudomonas* sp NIOCCAr27 (Chapter 3) and many other bacteria (Hespell and O'Bryan, 1992; Bezalel *et al.*, 1993; Morales *et al.*, 1995; Renner and Breznak, 1998; Degrassi *et al.*, 2003; Shin *et al.*, 2003; Kim *et al.*, 2004;

Birgisson *et al.*, 2004) and actinomycetes (Tajana *et al.*, 1992; Tuncer and Ball, 2003a).

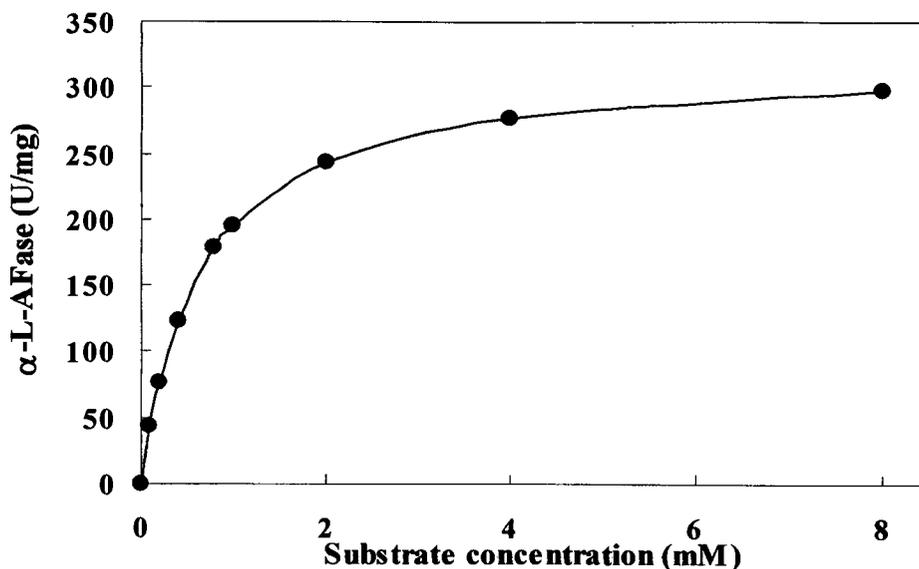


Fig. 4.2.6a: Effect of substrate concentration on the activity of  $\alpha$ -L-AFases produced by *Bacillus* sp NIOCCW19.

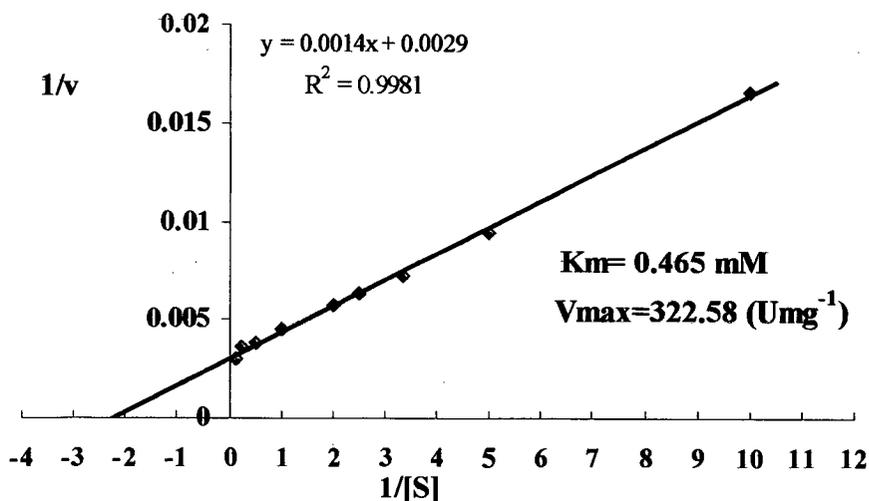


Fig. 4.2.6b: Double reciprocal plot for determination of the  $V_{max}$  and  $K_m$  values of  $\alpha$ -L-AFases against p-nitrophenyl- $\alpha$ -L-arabinofuranoside at 75°C.

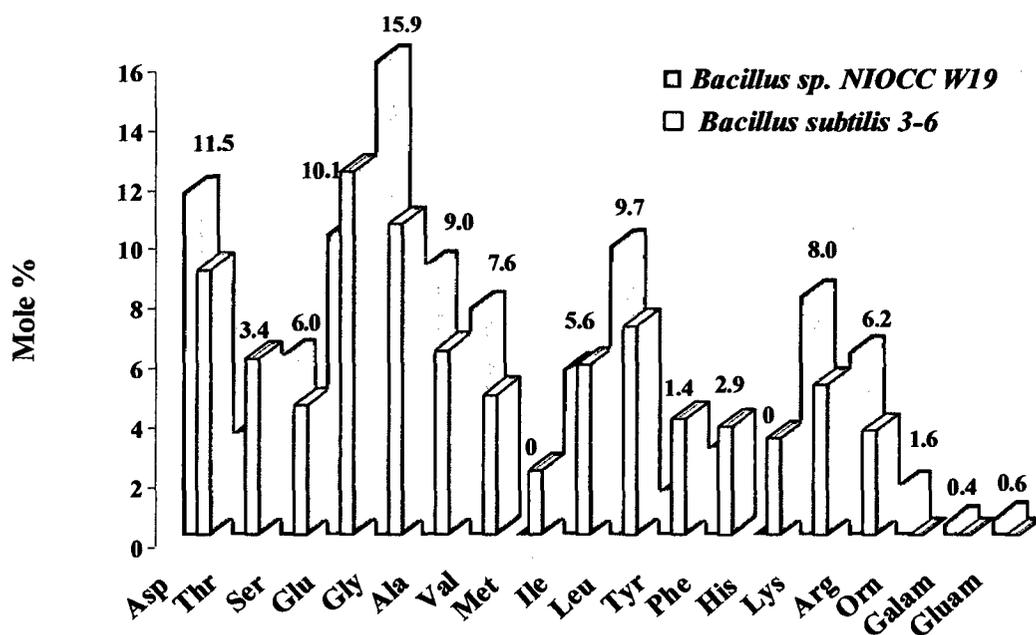
However, it was lower than that reported for  $\alpha$ -L-AFases from *B. stearothermophilus* T-6 (749  $Umg^{-1}$ ) (Gilead and Shoham, 1995), *Thermobacillus xylanilyticus* (555) (Debeche *et al.*, 2000), *Bifidobacterium*

*longum* B667 (417 U<sub>mg</sub><sup>-1</sup>) (Margolles and de los Reyes-Gavilán, 2003) and *Geobacillus caldoxylolyticus* TK4 (588.2 U<sub>mg</sub><sup>-1</sup>) (Canakci *et al.*, 2007). The  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 showed better affinity for the test substrate than that reported in some  $\alpha$ -L-AFases from several bacteria. This was evident from the low  $K_m$  value of  $\alpha$ -L-AFases of *Bacillus* sp NIOCCW19 compared to  $K_m$  values of  $\alpha$ -L-AFases reported from *Pseudomonas* sp NIOCCAr27 (Chapter 3) and many other bacteria (Greve *et al.*, 1984; Hespell and O'Bryan 1992; Renner and Breznak, 1998; Debeche *et al.*, 2000; Degrassi *et al.*, 2003; Kim *et al.*, 2004; Birgisson *et al.*, 2004) and some actinomycetes (Tajana *et al.*, 1992). Furthermore,  $K_m$  value of  $\alpha$ -L-AFases of *Bacillus* sp NIOCCW19 was similar to that reported for  $\alpha$ -L-AFases of *B. stearothermophilus* T-6 (Gilead and Shoham, 1995) and *Thermotoga maritime* (Miyazaki, 2005) and slightly higher than  $K_m$  value of  $\alpha$ -L-AFases from some bacteria (Bezalel *et al.*, 1993; Morales *et al.*, 1995; Margolles and de los Reyes-Gavilán, 2003; Shin *et al.*, 2003; Canakci *et al.*, 2007).

#### 4.2.3.6 Amino acid composition of $\alpha$ -L AFase

The amino acid analysis of  $\alpha$ -L-AFase from *Bacillus* sp.NIOCCW19 revealed that the enzyme contained 21.6 % and 15.21 % of acidic and basic amino acids, respectively (Fig. 4.2.7). It was reported previously that certain amino acids predominantly found in some microbial  $\alpha$ -L-AFases. These include amino acids such as aspartic acid, glutamic acid, glycine, and threonine (Kaneko *et al.*, 1994, Kimura *et al.*, 1995).  $\alpha$ -L-AFase from *Bacillus* sp was found to contain these amino acids too. However, the molar ratios of these amino acids in  $\alpha$ -L-AFase from *Bacillus* sp are different from those reported previously

(Kaneko *et al.*, 1994, Kimura *et al.*, 1995). Compare to  $\alpha$ -L-AFase from *Bacillus subtilis* 3-6 (Kaneko *et al.*, 1994), the  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 was found to have significantly higher molar ratio for certain amino acids. These included aspartic acid, serine, glycine, alanine, leucine, lysine and arginine. The enzyme also showed almost similar molar ratio of isoleucine and slightly lower molar ratio of glutamic acid, threonine, valine, tyrosine and phenylalanine (Fig. 4.2.7). The amino acid analysis of  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 showed that the enzyme contains more nonpolar (50.965 %) amino acids than polar (33.5 %) amino acids.



**Fig. 4.2.7:** Amino acid composition of  $\alpha$ -L-AFase from *Bacillus* sp. Amino acid composition of  $\alpha$ -L-AFase from *Bacillus subtilis* also given for comparison. Numbers in the figure are corresponding to the mole % of each amino acid present in  $\alpha$ -L-AFase from *Bacillus* sp.

The high thermostability of  $\alpha$ -L-AFase from *Bacillus* spNIOCCW19 could be attributed to this high content of nonpolar amino acids. It is well documented that the side chains of nonpolar and hydrophobic amino acids such as alanine, valine, leucine, isoleucine and glycine tend to cluster together within proteins,

stabilizing protein structure by means of hydrophobic interactions (Nelson and Lehninger, 2005). From a comparison of the amino acid composition of enzymes from thermophilic to mesophilic organisms, Argos *et al.* (1979) and Rüegg *et al.* (1982) proposed that thermostability is often caused by an increased amount of hydrophobic amino acid residues in the interior of the protein molecule. Among these hydrophobic amino acids is glycine which is present in unusual high molar ratio in  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19. The  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 could be a glycoprotein as it contains small molar ratios of N-glucosamine (0.6) and N-galactosamine (0.4). So far only one  $\alpha$ -L-AFase reported to be glycoprotein which was isolated from *Ruminococcus albus* 8 (Greve *et al.*, 1984). Furthermore, *R. albus*  $\alpha$ -L-AFase is a glycoprotein evident by the presence of glucosamine in the carbohydrate of the protein (Greve *et al.*, 1984). The  $\alpha$ -L-AFase from *Streptomyces diastatocheomogenes* 065 was reported to have associated carbohydrates (Higashi *et al.*, 1983). Moreover, some hemicellulases such as xylanases from prokaryotic sources, like *Clostridium stercorarium* (Berenger *et al.*, 1985), *Streptomyces* sp (Marui *et al.*, 1985) and an alkaliphilic and thermophilic *Bacillus* sp (Dey *et al.*, 1992), were found to be glycoproteins. Carbohydrate groups are covalently linked with protein or are present as dissociable complexes with these enzymes (Kulkarni *et al.*, 1999). The amount of polysaccharides attached to a given glycoprotein can vary enormously, from as little as a few percent to more than 60% by weight. The precise role played by the carbohydrate moiety of glycoproteins includes stabilization of the protein structure, protection of the protein from degradation by proteases, control of protein half-life and as an important determinant in substrate-ligand binding.

Moreover, glycosylation can affect the half-life, stability and function of the protein (Wilson and Walker, 2005). Thus, the high thermostability of  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 could be attributed to the presence of aminosugar in the enzyme structure. In fact, glycosylation has been implicated in the stabilization of glycanases against extreme environments (Merivuori *et al.*, 1985). For example, the recombinant xylanases expressed in *Escherichia coli* from an alkaliphilic thermophilic *Bacillus* sp (Kulkarni *et al.*, 1995) showed lower stability at higher temperature and reduced ability to bind xylan compared to xylanases from the parent strain which is attributed to deglycosylation (Kulkarni *et al.*, 1999).

#### 4.2.3.7 Effect of metal ions and other chemical agents on $\alpha$ -L AFase

The effect of certain inhibitors or activators on the activity of  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 was studied (Table 4.2.3).

**Table 4.2.3:** Effect of metal ions and chemical agents on  $\alpha$ -L-AFase activity

<b>Chemical agent (1mM)</b>	<b>Residual activity (%)</b>
<b>Non</b>	<b>100.00</b>
<b>Ca<sup>2+</sup></b>	<b>98.14</b>
<b>Co<sup>2+</sup></b>	<b>99.57</b>
<b>Cu<sup>2+</sup></b>	<b>77.92</b>
<b>Fe<sup>3+</sup></b>	<b>107.84</b>
<b>Hg<sup>2+</sup></b>	<b>57.88</b>
<b>Mg<sup>2+</sup></b>	<b>101.80</b>
<b>Mn<sup>2+</sup></b>	<b>103.64</b>
<b>Zn<sup>2+</sup></b>	<b>93.75</b>
<b>DTT (10 mM)</b>	<b>99.48</b>
<b><math>\beta</math>-mercaptoethanol (5mM)</b>	<b>77.18</b>
<b>EDTA</b>	<b>99.85</b>
<b>SDS</b>	<b>81.78</b>
<b>L-arabinose</b>	<b>99.14</b>

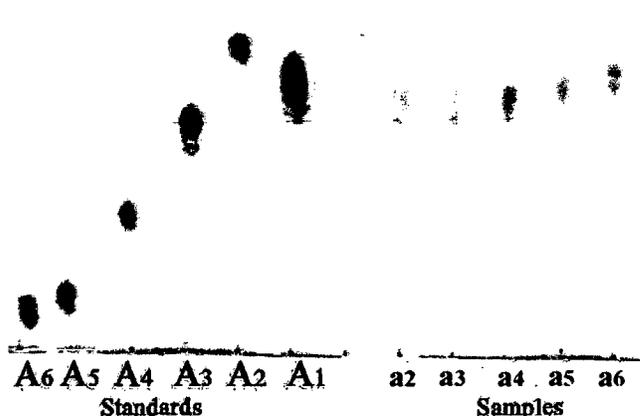
The presence of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , or  $\text{Co}^{2+}$  did not enhance enzyme activity, and slight increase in the enzyme activity was observed in the presence of  $\text{Fe}^{2+}$  and  $\text{Mn}^{2+}$ . However, presence of EDTA did not affect enzyme activity. This may suggest that  $\alpha$ -L-AFase from *Bacillus* sp needs no metals to carry out the hydrolysis reaction. Furthermore, presence of L-arabinose (up to 100mM) has no major effect on the enzyme activity, suggesting that no end-product inhibition has taken place in the presence of L-arabinose. Similar observation was reported for  $\alpha$ -L-AFase from *Streptomyces lividans* 66 (Manin *et al.*, 1994). However, L-arabinose inhibited the activity of  $\alpha$ -L-AFase from *Phytophthora palmivora* (Burt) (Akinrefon, 1968) and *Aureobasidium pullulans* (De Wet *et al.*, 2008). According to De Wet *et al.* (2008) L-arabinose acted as a noncompetitive inhibitor for  $\alpha$ -L-AFase from *Aureobasidium pullulans*, which binds at a site separate from the active site to exert its inhibitory effect. As L-arabinose did not inhibit the activity of  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19, this may suggest the absence of such noncompetitive inhibitor binding site in this enzyme. The reducing agent dithiothreitol (DTT) showed no effect on the enzyme activity indicating that disulfide bonds are not critical for enzyme activity. However,  $\beta$ -mercaptoethanol and SDS caused moderate inhibition of enzyme activity. Conversely, both these chemicals cause severe inhibition to bacterial and fungal  $\alpha$ -L-AFases (Uesaka *et al.*, 1978; Greve *et al.*, 1984; Riou *et al.*, 1991 ; Fernández-Espinar *et al.*, 1994 ;Gilead and Shoham, 1995; Filho *et al.*, 1996; Le Clinche *et al.*, 1997; Debeche *et al.*, 2000; Tuncer, 2000; Yanai and Sato, 2000; Kosugi *et al.*, 2002; Takao *et al.*, 2002; Tsujibo *et al.*, 2002; Margolles and de los Reyes-Gavián, 2003; Sakamoto *et al.*, 2003; Oshima *et al.*, 2005). Moreover, addition of  $\text{Hg}^{+2}$  caused a

moderate inhibition in the enzyme activity (**Table 4.2.3**). However, severe inhibition was recorded in the activity of  $\alpha$ -L-AFases isolated from many microorganisms (Gilead and Shoham, 1995; Saha and Bothast, 1998b; Le Clinche *et al.*, 1997; Debeche *et al.*, 2000; Yanai and Sato, 2000; Kosugi *et al.*, 2002; Margolles and de los Reyes-Gavilán, 2003; Shin *et al.*, 2003; Miyazaki, 2005; Khandeparker *et al.*, 2008).

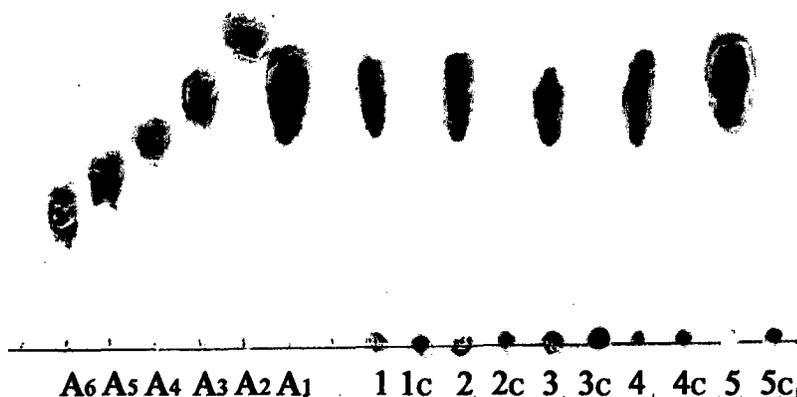
#### **4.2.3.8 Substrate specificities of $\alpha$ -L AFase**

The substrate specificity of the  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 was tested with a series of aryl-p-nitrophenyl derivatives including *p*NP- $\alpha$ -L-arabinofuranoside, *p*NP- $\beta$ -D-xylopyranoside, *p*NP- $\alpha$ -L-arabinopyranoside, *o*NP- $\beta$ -D-xylopyranoside, *p*NP- $\beta$ -D-glucopyranoside, *p*NP- $\alpha$ -D-galactopyranoside and *p*NP- $\beta$ -D-galactopyranoside. The enzyme showed activity on *p*NP- $\alpha$ -L-arabinofuranoside and no activity was detected against other substrates indicating high specificity for the  $\alpha$ -L-arabinofuranosyl linkage. The enzyme released only arabinose from arabinooligosaccharides, arabinan and debranched arabinan as detected by thin layer chromatography (**Plate 4.2.2a** and **Plate 4.2.2b**). As no arabinobiose or higher degree oligomer was detected, this may indicate that the enzyme acts by exo-acting manner. The enzyme behavior in releasing arabinose from branched and debranched arabinan was surprising. This is because none of the reported  $\alpha$ -L-AFases from genus *Bacillus* and its related species was able to act on both of these substrates. Moreover, such enzymatic activity was reported in few  $\alpha$ -L-AFases which included  $\alpha$ -L-AFases from *Corticium rolsii* (Kaji and Yoshihara, 1971),

*Streptomyces* sp. strain 17-1 (Kaji *et al.*, 1981), *Aspergillus niger* (Rombouts *et al.*, 1988), *Streptomyces diastaticus* (Tajana *et al.*, 1992), *Streptomyces lividans* 66 strain 1326 (Manin *et al.*, 1994), Bacterium PRI-1686 (Birgisson *et al.*, 2004) , *T. maritima* MSB8 (Miyazaki, 2005) and *Pseudomonas* sp NIOCCAr27 (Chapter 3).



**Plate 4.2.2a:** TLC analysis of the hydrolysis products released from arabinofuranooligosaccharides by *Bacillus* sp.  $\alpha$ -L-AFase. Standards include: **A1:** arabinose, **A2:** arabinobiose, **A3:** arabinotriose, **A4:** arabinotetraose, **A5:** arabinopentaose and **A6:** arabinohexaose. Samples include: **a2:** arabinobiose, **a3:** arabinotriose; **a4:** arabinotetraose, **a5:** arabinopentaose and **a6:** arabinohexaose.



**Plate 4.2.2b:** TLC analysis of the hydrolysis products released from arabinofuranopolysaccharides natural substrates by *Bacillus* sp.  $\alpha$ -L-AFase. The substrates included: **1:**Wheat arabinoxylan, **2:** Oat spelt xylan, **3:** Arabinogalactan, **4:** Debranched arabinan and **5:** Arabinan. The Standards: **A1:** arabinose, **A2:** arabinobiose, **A3:** arabinotriose, **A4:** arabinotetraose, **A5:** arabinopentaose, **A6:** arabinohexaose. The controls: **1c:**Wheat arabinoxylan, **2c:** Oat spelt xylan, **3c:** Arabinogalactan, **4c:** Debranched arabinan and **5c:** Arabinan.

Liberation of arabinose was detected by thin layer chromatography when enzyme was incubated with oat spelt xylan, wheat arabinoxylan, or arabinogalactan (**Plate 4.2.2b**). Other than arabinose, no carbohydrate moieties were detected after  $\alpha$ -L-AFase treatment of these substrates, indicating that this enzyme probably functions as a specific exoenzyme. The results showed that the enzyme acted on all polymeric substrates tested. Similar enzymatic activity on some but not all of these substrates has been described for family 54 arabinofuranosidases (Henrissat and Bairoch, 1996; Beldman *et al.*, 1997).

Whereas, the nature of a glycone sugar can influence the catalytic activity of other  $\alpha$ -L-AFases isolated from different microorganisms (Beldman *et al.*, 1997; Saha, 2000). The enzyme from *Bacillus* sp hydrolyzed  $\alpha$ -L-arabinofuranosyl moieties attached to any sugar in the polymeric substrates tested. This may suggest that, the nature of a glycone sugar in these substrates has no determinative action on the enzyme activity. It also suggest that the enzyme acts on all types of  $\alpha$ -L arabinofuranosyl linkages including  $\alpha$ -L-1,2 ;  $\alpha$ -L-1,3 and  $\alpha$ -L-1,5 linkages. Thus, the  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 was found to have wide substrates specificity. In contrast,  $\alpha$ -L-AFase from *Pseudomonas* sp NIOCCAr27 ( **Chapter 3**) was active only on branched arabinan and linear arabinan and arabinooligosaccharides. Furthermore, the  $\alpha$ -L-AFase from *Pseudomonas* sp was specific for hydrolysis of (1-3)- $\alpha$ -L- and (1-5)- $\alpha$ -L- arabinofuranosyl branch units present in arabinan and debranched arabinan.

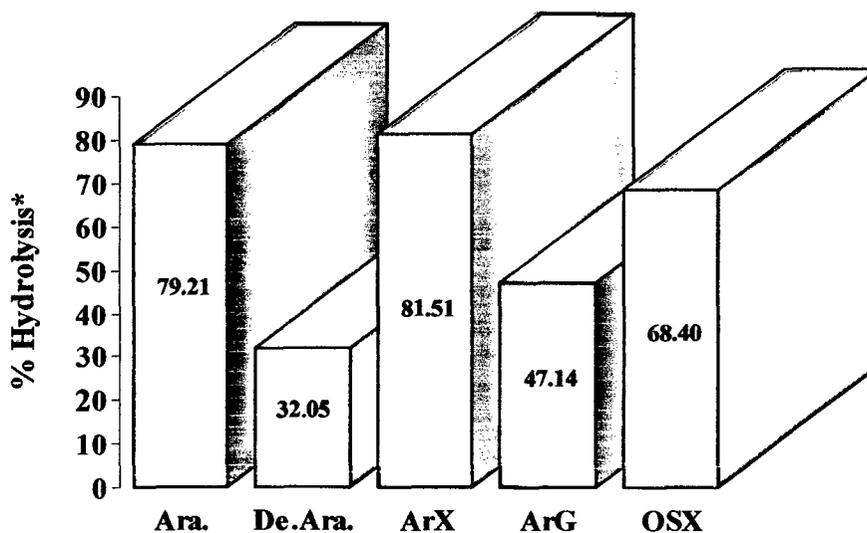
Defining a common range of substrate specificity for  $\alpha$ -L-AFases is a difficult task. This is because of the differences in methodology and substrates used to study these enzymes. For examples *p*NPAF, arabinooligosaccharides, arabinan,

debranched arabinan, arabinoxylan, arabinogalactan and oat spelt xylan were among substrates used to study substrate specificity of  $\alpha$ -L-AFases from different bacteria. These include *Streptomyces* sp strain 17-1 (Kaji *et al.*, 1981), *Streptomyces purpuracens* IFO 3389 (Komae *et al.*, 1982), *Erwinia carotovora* IAM 1024 (Kaji and Shimikawa, 1984), *Ruminococcus albus* 8 (Greve *et al.*, 1984), *Clostridium acetobutylicum* ATCC824 (Lee and Forsberg, 1987), *Clostridium stercorearium* (Schwarz *et al.*, 1990), *Thermomonospora fusca* (Bachmann and McCarthy, 1991), *Butyrivibrio fibrisolvens* GS113 (Hespell and O'Bryan, 1992), *Streptomyces diastaticus* (Tajana *et al.*, 1992), *Clostridium stercorearium* (Sakka *et al.*, 1993), *Streptomyces lividans* 66 (Manin *et al.*, 1994), *Bacteroides xylanolyticus* X5-1 (Schyns *et al.*, 1994), *S. chartreusis* GS901 (Matuso *et al.*, 2000), *P. cellulosa* (Beylot *et al.*, 2001b), *Clostridium cellulovorans* (Kosugi *et al.*, 2002), *Streptomyces thermavioles* OPC-520, (Tsujiho *et al.*, 2002), *Bifidobacterium longum* B667 (Margolles and de los Reyes-Gavilán, 2003), Bacterium PRI-1686 (Birgisson *et al.*, 2004), *T. maritima* MSB8 (Miyazaki, 2005) and *Pseudomonas* sp NIOCCAr27 (Chapter 3). However,  $\alpha$ -L-AFases from these bacteria were able to release arabinose from some of these substrates. Whereas, all the  $\alpha$ -L-AFases from these bacteria were not active on all of these substrates. Interestingly,  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 was able to release arabinose from all these substrates. This may suggest that the  $\alpha$ -L-AFases from *Bacillus* sp NIOCCW19 has a broader range of substrates specificity than most of  $\alpha$ -L-AFase reported from other bacteria including *Pseudomonas* sp NIOCCAr27 (Chapter 3). Moreover, as compared to  $\alpha$ -L-AFases isolated from

other strains of *Bacillus*, the  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 showed a broader range of substrates specificity (Table 4.2.2).

#### 4.2.3.9 Hydrolysis ratio of the substrates

Figure 4.2.8 shows the levels of hydrolysis of arabinan, debranched arabinan, wheat arabinoxylan, arabinogalactan, and oat spelt xylan by the  $\alpha$ -L-AFases from *Bacillus* sp NIOCCW19. The enzyme produced different amounts of arabinose from these substrates. The amount of arabinose produced by enzymatic action on branched arabinan was more than that produced from debranched arabinan. This may suggest that the enzyme preferentially hydrolyzed the monosaccharide arabinofuranosyl side chains of arabinan. It may also suggest that the enzyme hydrolyzed debranched arabinan slowly from the nonreducing terminus.



**Fig. 4.2.8:** Action of  $\alpha$ -L-AFase on some hemicelluloses measured as the release of arabinose from Ara: Arabinan; De.Ara.:Debranched Arabinan; ArX.:wheat Arabinoxylan,ArG: Arabinogalactan and OSX: Oat spelt xylan.

\* Levels of hydrolysis were estimated according to Kaneko *et al.* (1998b). Numbers in the figure indicated the level of hydrolysis of each substrate.

The enzyme behavior in releasing more arabinose from branched arabinan than that from debranched arabinan is not surprising. Similar behavior of  $\alpha$ -L-AFase on arabinan and debranched arabinan was reported for  $\alpha$ -L-AFases from *Corticium rolfsii* (Kaji and Yoshihara, 1971), *Streptomyces* sp strain 17-1 (Kaji *et al.*, 1981), *Aspergillus niger* (Rombouts *et al.*, 1988), *Streptomyces diastaticus* (Tajana *et al.*, 1992), *Streptomyces lividans* 66 strain 1326 (Manin *et al.*, 1994), and Bacterium PRI-1686 (Birgisson *et al.*, 2004).

When incubated with arabinogalctan, the  $\alpha$ -L-AFase generated monosaccharide arabinose that accounted for about 47.14% of the arabinose available in arabinogalactan. However, the  $\alpha$ -L-AFase generated significantly larger proportions of monosaccharide arabinose when incubated with wheat arabinoxylan and oat spelt xylan (Fig. 4.2.8). This may suggest that the enzyme is more efficient in hydrolysis of  $\alpha$ -L-1,3, and  $\alpha$ -L-1,5 arabinofuranosyl linkages present in arabinoxylan and oat spelt xylan than  $\alpha$ -L-1,2 arabinofuranosyl linkages present in arabinogalactan.

The extent of substrates hydrolysis achieved by  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 was higher than that achieved by any  $\alpha$ -L-AFases from other microorganisms (Bachmann and McCarthy, 1991; Kimura *et al.*, 1995; Wood and McCrae, 1996; Kaneko *et al.*, 1998a;b;c; Renner and Breznak, 1998; Margolles and de los Reyes-Gavilán, 2003; Kim *et al.*, 2004; De Wet *et al.*, 2008). The high activity of  $\alpha$ -L-AFase from *Bacillus* sp NIOCCW19 on broad range of hemicellulosic substrates at broad range of temperature and pH proved the superiority of this enzyme. Furthermore, these properties make the enzyme

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# *Chapter 5*

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**Cooperative Action and Application of  $\alpha$ -L-Arabinofuranosidase****Produced by *Bacillus* spNIOCCW19****5.1.1 INTRODUCTION**

Lignocellulosic biomass is a constant source of sustainable and renewable energy in earth (Mantanis, 1999). Lignocelluloses contain typically cellulose (35-55 %), hemicellulose (20-40 %) and lignin (10-25 %). One of the first requirements for efficient utilization of lignocelluloses is the hydrolysis of lignocellulosic cellulose and hemicellulose into their hexose and pentose sugars. This can be accomplished by the cooperative action of cellulases and hemicellulases such as  $\alpha$ -L-arabinofuranosidase and xylanase (Boisset *et al.*, 1998; Saha, 2000; Sørensen *et al.*, 2003;2005; Koukiekolo *et al.*, 2005; Sørensen *et al.*,2006a,b;). This enzyme based process is an attractive approach to obtain fermentable sugars that can be used for industrial processes such as bioethanol production (Roehr, 2001; Schell *et al.*, 2003, Silverstein *et al.*, 2007). These sugars can be converted into ethanol using genetically engineered microorganisms such as *Zymomonas mobilis* and *Escherichia coli* (Bothast *et al.*, 1994; Dien *et al.*, 2000; Roehr, 2001). Moreover, utilization of lignocellulosic biomass represents a vital solution to reduce the cost of fermentable sugars production used in bioethanol industry (Ghosh and Ghose, 2003; Chang and Holtzaple, 2000; Gray *et al.*, 2006). Further cost reductions are required and will more likely come from inexpensive, tailored cocktails of enzymes with higher specific activities than current commercial enzymes (Gray *et al.*, 2006; Sørensen *et al.*, 2006a;b). Lignocelluloses, however, are extremely recalcitrant, and their utilization for sugars production requires suitable

pretreatment (Cadoche and Lopez, 1989; Gregg and Saddler, 1996; Kuhad *et al.*, 1997; Keller *et al.*, 2003; Kim *et al.*, 2003; Damaso *et al.*, 2004; Chandel *et al.*, 2007). The ideal pretreatment must remove lignin, improve the availability of sugars, prevent degradation of carbohydrate (hemicellulose and cellulose) into non-sugars compounds, reduce unfavorable and inhibitory by-products, and be low cost (Sun and Cheng, 2002).

Sugar cane is one of the most abundant crops in India. Apart from being invaluable for sugar production, the bagasse produced from sugar cane is also a significant source of lignocellulosic biomass (Ghosh and Ghose, 2003). For efficient utilization of sugar cane bagasse for fermentable sugars production, suitable pretreatment is required to render the cellulose and hemicelluloses more amenable to the action of hydrolytic enzymes. Therefore, this study was initiated to assess the potential of the cooperative action of  $\alpha$ -L-AFase and xylanase produced by *Bacillus* sp NIOCCW19 in degradation of some hemicelluloses. Furthermore, an attempt has been made to develop suitable chemo-enzymatic treatment which provides the highest biomass to reducing sugars conversion during subsequent hydrolysis with enzyme cocktails that includes cellulases, xylanase and  $\alpha$ -L-AFase.

## **5.1.2 MATERIALS and METHODS**

### **5.1.2.1 Biomass:**

Sugar cane bagasse was collected locally, washed with water, oven dried at 80°C to a constant weight. It was then grinded using a commercial coffee grinder and sieved through 40 mesh sieve. This sieved bagasse was stored in sealed container at room temperature until use for pretreatment experiments.

### 5.1.2.2 Enzymes

$\alpha$ -L-Afase from *Bacillus* sp NIOCCW19 was assayed as described in Section 1 of the Chapter 4. Xylanase from *Bacillus* sp NIOCCW19 was assayed as described in Chapter 2.

Cellulase preparation from *Trichoderma reesei* was procured from Sigma-Aldrich Co. Cellulase activity was measured according to National Renewable Energy Laboratory (NREL) procedure (Sluiter *et al.*, 2004). This was done by using 0.5 ml appropriately diluted enzyme in citrate buffer pH 5.5, 50 mg Whatman No. 1 filter papers strip and 1.0 ml 50 mM Na-citrate pH 5.5. The mixture was incubate at 50°C for 60 min. At the end of the incubation period, 3.0 ml DNS reagent was added to stop the enzyme reaction. The released sugar was determined after subtracting the reading of the control from the sample reading using a glucose standard curve. The enzyme activity was expressed in Filter Paper Units (FPU/g solid) according to an NREL procedure (Sluiter *et al.*, 2004).

The  $\beta$ -glucosidase from *Aspergillus niger* was procured from Sigma-Aldrich Co. The  $\beta$ -glucosidase was assayed following the method described by Saha *et al.* (2005). The reaction mixture (1 ml) contained 4 mM *p*-nitrophenyl- $\beta$ -D-glucoside, 50 mM acetate buffer, pH 5.5; and appropriately diluted enzyme solutions. After incubation at 50 °C for 30 min, the reaction was stopped by adding 1 ml of ice-cold 0.5 M Na<sub>2</sub>CO<sub>3</sub>, and the color that developed as a result of *p*-nitrophenol liberation was measured at 405 nm. One unit (U) of  $\beta$ -glucosidase activity is defined as the amount of enzyme that releases 1  $\mu$ mol of *p*-nitrophenol per min in the reaction mixture under these assay conditions.

### **5.1.2.3 Evaluation of the cooperative action of $\alpha$ -L-AFase in degradation of hemicellulosic arabinoxylans**

The  $\alpha$ -L-AFase from *Bacillus* sp was assessed for its contribution to both wheat arabinoxylan and oat spelt xylan hydrolysis in the presence and absence of xylanase purified from same bacterium. This was done following the method described by Renner and Breznak (1998). Sodium phosphate buffer (50 mM), pH 8.0, containing either wheat arabinoxylan (1%) and oat spelt xylan (0.5%) was placed in conical flasks. To these flasks either  $\alpha$ -L-AFase (0.5 U) or xylanase (20 U) or combination of  $\alpha$ -L-AFase (0.5 U) and xylanase (20 U) was added. Flasks with 50 mM sodium phosphate buffer, pH 8.0, and substrate were used as control. The flasks were incubated on a rotary shaker (80 RPM) at 50°C for 24 h. The flasks content were heated in water bath for 10 min and centrifuged at 10,000 RPM for 10 min. The reducing sugars released in the supernatants were estimated as xylose equivalents using Somogyi- Nelson method (1952).

### **5.1.2.4 Determination of potential sugar yield of bagasse**

For determination of the potential maximum yield of carbohydrate (total sugar) ( $TS_{max}$ ) that can be obtained from bagasse, dried bagasse was treated according to the NREL standard procedure (Belkacemi *et al.*, 1997; Sluiter *et al.*, 2004). This procedure represented by the two-stage acid hydrolysis of dry bagasse. In the first stage bagasse sample (150 mg) was treated with 72% (w/w)  $H_2SO_4$  (1.5 ml) at 30°C for 1 h. In the second stage (post hydrolysis), the solution was diluted to 7.2 % (w/w)  $H_2SO_4$  with distilled water (42 ml) and autoclaved at 121°C for 1 h. After cooling to room temperature the hydrolysates were filtered through Whatman No. 1 filter papers under vacuum. Solid fractions on the filter

paper were washed thoroughly with distilled water under vacuum and oven dried at 80°C for 24 h and the weight was determined. This represents the amount of lignin and ash in bagasse sample. The filtrate (15ml) was neutralized with 6N NaOH and brought to 100 ml with distilled water. The total (carbohydrate) sugar content ( $TS_{max}$ ) in the filtrate was estimated by phenol-sulphuric acid method (Dubois *et al.*, 1956) and glucose was used as standard. The free reducing sugars initially present in bagasse ( $FS_i$ ) was determined by the 3,5-dinitrosalicylic acid method (DNS) (Miller, 1959) and glucose was used as standard.

#### **5.1.2.5 Pretreatments of sugar cane bagasse**

##### **5.1.2.5A Chemical pretreatments of sugar cane bagasse**

Three chemical pretreatment of sugar cane bagasse were conducted that included:

###### **5.1.2.5A.1 Diluted acid treatment:**

Bagasse was treated with hot diluted HCl following the method described by Kurakake *et al.* (2005). Bagasse sample (10 g) and 90 ml of 0.2 N HCl was mixed in glass container. The container was then capped and heated at 130°C for 30 min in an oven. After heating they were cooled down to room temperature and neutralized with 1N NaOH. The pH value of the formed mixture was adjusted to pH 5.5 or pH 8.0 as required by adding 100 mM acetate buffer (pH 5.5) or 50 mM Na-phosphate buffer (pH 8.0), respectively.

###### **5.1.2.5A.2 Hydrogen peroxide treatment**

Hydrogen peroxide treatment of bagasse was conducted following the method described by Silverstein *et al.* (2007). Bagasse sample (10 g) was mixed with (90 ml) of 2% hydrogen peroxide in capped flask and was autoclaved at 121°C and 15 psi for 90 min. The mixture was cooled to room temperature and

neutralized with 6N HCl. The pH value of the mixture was then adjusted to pH 5.5 or pH 8.0 as described above.

#### **5.1.2.5A.3 Alkaline treatment**

Alkaline treatment was conducted using NaOH following the method described by Chen *et al.* (2008). Bagasse sample (10 g) and (90ml) of 2% NaOH was heated at 80°C for 1.5 h. This was cooled to room temperature and neutralized using 6N HCl. The pH value of the mixture was then adjusted to pH 5.5 or pH 8.0 as described above.

One sample of either of acid, H<sub>2</sub>O<sub>2</sub> and NaOH pretreated bagasse was processed to separate pretreated bagasse from the soluble sugars. Pretreated bagasse sample was transferred to a glass beaker and distilled water was added and the sample was stirred for 1 h using magnetic stirrer. After stirring, the bagasse sample was filtered under vacuum and rinsed thoroughly using distilled water. The volume of the filtrate obtained was measured with measuring cylinder and recorded. The filtrate was used for determination of the total free sugars (TS<sub>C</sub>) released by chemical treatments. The amount of reducing sugars was estimated by the dinitrosalysilic acid (DNS) method (Miller, 1959) using glucose as a standard.

#### **5.1.2.5B Enzymatic treatments:**

Enzymatic hydrolysis of chemically pretreated bagasse samples was carried out at 50°C in erlenmeyer flasks placed on a rotary shaker at 100 RPM for the required periods of time described below. The enzymes used for hydrolysis of chemically-pretreated bagasse included  $\alpha$ -L-AFase (5U/g), xylanase (80U/g), cellulase (20FPU/g) and  $\beta$ -glucosidase (4U/g). Sodium azide at 0.005% (w/w) was added to keep the sample sterile during enzyme treatments. Both the

enzymes cellulase and  $\beta$ -glucosidase are referred hereafter as cellulases. Enzymatic treatments were conducted after adjustment of pH of the reaction mixture as describe above. Different enzymatic treatments were conducted as described below:

**5.1.2.5B.1 Individual enzyme treatment:**

- a.  $\alpha$ -L-AFase treatment at pH 8 for 72 h.
- b. Xylanase treatment at pH 8 for 72 h.
- c. Cellulases treatment at pH 5.5 for 72 h.

**5.1.2.5B.2 Treatment by using mixture of  $\alpha$ -L-AFase and xylanase at pH 8 for 24 h.**

**5.1.2.5B.3 Sequential treatments:**

- a.  $\alpha$ -L-AFase treatment at pH 8 for 24 h followed by xylanase treatment at pH 8 for 24 h.
- b.  $\alpha$ -L-AFase treatment at pH 8 for 24 h followed by adjusting the pH to 5.5 and treating with cellulases for 72 h.
- c. Treating with xylanase at pH 8 for 24 h followed by adjusting the pH to 5.5 and treating with cellulases for 72 h.
- d. Treating together with the mixture of  $\alpha$ -L-AFase and xylanase at pH 8 for 24 h followed by adjusting the pH to 5.5 and treating with cellulases for 72 h.

**5.1.2.5B.4 Treatment using mixture of  $\alpha$ -L-AFase, xylanase and cellulases at pH 5.5 for 96 h.**

After 24 h intervals of enzymatic hydrolysis, samples were withdrawn and placed in a boiling water bath for 15 min to deactivate the enzymes. This was then passed through a 0.2  $\mu$ m filter paper under vacuum. A portion of 0.2 ml

was used for measurement of the total reducing sugars (TS<sub>E</sub>) by the dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose as standard.

The efficiency (or yield) of either of chemical treatments, enzyme treatments or both treatments was calculated according to (Belkacemi *et al.*, 1997, 2002). The efficiency (yield) of either chemical treatments or enzymatic treatments was expressed as a percentage of saccharification (%) and calculated using Eq. 1 and Eq. 2, respectively.

$$\text{Chemical Saccharification (\%)} = [(TS_C - FS_I) / (TS_{max} - FS_I)] \times 100 \quad (\text{Eq. 1})$$

$$\text{Enzymatic Saccharification (\%)} = [(TS_E - FS_I) / (TS_{max} - FS_I)] \times 100 \quad (\text{Eq. 2})$$

where,

TS<sub>max</sub>: The potential maximum yield of sugars released after two stage acid hydrolysis of bagasse, FS<sub>I</sub>: Free sugars initially present in bagasse TS<sub>C</sub>: total sugars chemically released from the substrate and TS<sub>E</sub>: total sugars enzymatically released from pretreated substrate. In most cases, FS<sub>I</sub> was found to be negligible.

The efficiency of whole saccharification processes was expressed as percentage of saccharification (%) and calculated as the sum of chemical treatment and enzymatic treatment, over the potential maximum yield of total sugars (TS<sub>max</sub>) (Equation 3)

$$\text{Total Saccharification (\%)} = [(TS_E + TS_C) / (TS_{max})] \times 100 \quad (\text{Eq.3}) \quad \text{where,}$$

TS<sub>max</sub>: The potential maximum yield of total sugars released after two stage acid hydrolysis of bagasse, TS<sub>C</sub>: total free sugars released by chemical treatment of bagasse and TS<sub>E</sub>: total sugars enzymatically released from pretreated substrate.

Each experimental value in this section represented the average of two independent determinations. The standard deviations (SD $\pm$ ) of these readings were equal or less than 10 % of the calculated means.

### **5.1.3 RESULTS and DISCUSSION**

#### **The cooperative action of $\alpha$ -L-AFase in degradation of hemicellulosic arabinoxylans**

The hemicellulosic arabinoxylans are readily hydrolyzed to monosaccharides by acid treatment. In the industrial upgrading of arabinoxylans, however, enzymatic hydrolysis is preferable. This is mainly because enzymatic hydrolysis allows specific, controlled modifications, avoids generation of undesirable by-products, and generally provides an environmental friendly process (Sheehan *et al.*, 1999, Saha, 2004; Sørensen *et al.*, 2006a;b). As a consequence of the complex branching and heterogeneous composition of arabinoxylans, their enzymatic degradation requires action of both debranching and depolymerizing activities. The  $\alpha$ -L-AFase represents a debranching, whereas xylanase represents the depolymerizing enzyme (Saha, 2000). In arabinoxylans, however, arabinose represents the major side chains sugar and arabinose:xylose ratio in arabinoxylans is up to 0.67 and some times exceeds this value (Sørensen *et al.*, 2006b). Therefore, removal of arabinose side chains by  $\alpha$ -L-AFase greatly enhances the action of xylanase for complete degradation of arabinoxylans (Sørensen *et al.*, 2003;2005;2006a;b). It has been found that *Bacillus* sp NIOCCW19 co-produces good amounts of  $\alpha$ -L-AFase and xylanase when grown on cheap agricultural residues under SmF and SSF (Chapter 2). Due to the importance of the cooperative action of  $\alpha$ -L-AFase and xylanase and

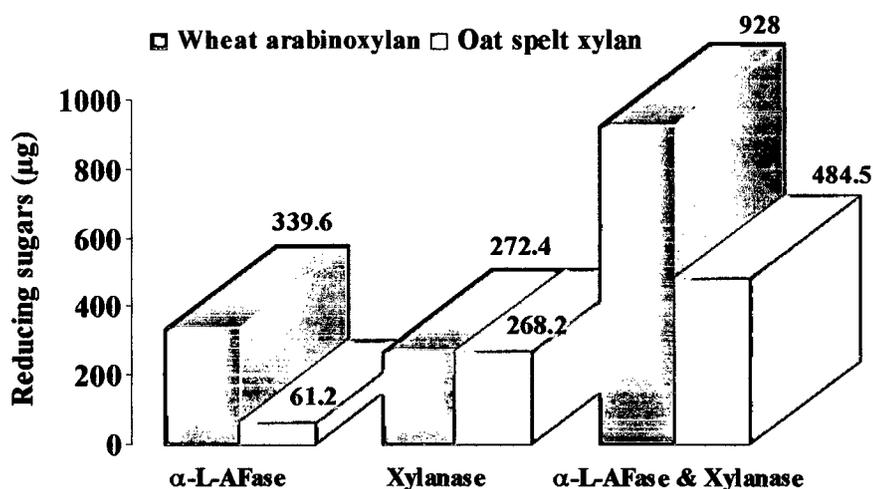
because for most of the industrial applications simultaneous use of xylanase and  $\alpha$ -L-AFase is preferred, it was thought worthwhile to study the synergetic effect of  $\alpha$ -L-AFase and xylanase on some hemicelluloses arabinoxylans. The properties of xylanase from *Bacillus* sp NIOCCW19 is given in **Table 5.1.1**.

**Table 5.1.1:** Biochemical properties of xylanase from *Bacillus* sp NIOCCW19.

<b>Biochemical Property</b>	<b>Remarks</b>
Optimum temperature	80°C.
Activity at 50°C	68.69 % of original activity at its optimum temperature
Stability at 50°C	100 % Stable for more than 24 h.
Optimum pH	9.0.
Activity at pH 8	96.70 % of the original activity at its optimum pH.
Activity at pH 5.5	64.1 % of the original activity at its optimum pH.
Stability at pH 8	100 % Stable for more than 24 h.
Stability at pH 5.5	Retain 51.13% of its original activity for 24 h.

In order to understand the actual contribution of  $\alpha$ -L-AFase of *Bacillus* sp NIOCCW19 to the hydrolysis of arabinoxylan containing substrates, the enzyme action on these substrates was studied in the presence and absence of xylanase obtained from the same bacterium. The changes in hydrolysis rates caused by the  $\alpha$ -L-AFase in both cases were determined by estimating the release of xylose equivalents from the arabinoxylan substrates. It was found that,  $\alpha$ -L-AFase alone released 339.6  $\mu$ g/ml and 61.2  $\mu$ g/ml reducing sugars from wheat arabinoxylan and oat spelt xylan, respectively (**Fig. 5.1.1**). Further, xylanase alone released 272.4  $\mu$ g/ml and 268.2  $\mu$ g/ml reducing sugars from wheat arabinoxylan and oat spelt xylan, respectively (**Fig. 5.1.1**). When both enzymes  $\alpha$ -L-AFase and xylanase applied together, 928  $\mu$ g/ml and 484.5  $\mu$ g/ml

reducing sugars were released from wheat arabinoxylan and oat spelt xylan, respectively (Fig. 5.1.1). The enhancement in the substrates hydrolysis represent the increase in the amount of reducing sugars released compared with the amount expected from the action of  $\alpha$ -L-AFase alone or xylanase alone (Renner and Breznak, 1998). It was observed that presence of  $\alpha$ -L-AFase in combination with xylanase caused enhancement of about 51.63 % and 47.09 % in releasing reducing sugars from wheat arabinoxylan and oat spelt xylan, respectively (Fig. 5.1.1).



**Fig. 5.1.1:** Cooperative action of  $\alpha$ -L-AFase and xylanase in hydrolysis of wheat arabinoxylan and oat spelt xylan. Numbers in the figure are corresponding to amount of reducing sugar obtained by enzymes action.

The increased rates of hydrolysis of these substrates was apparently due to catalytic synergy, as apposed to enhanced activity of xylanase conferred by the presence of  $\alpha$ -L-AFase. Moreover, it seems that removal of the arabinose side chains by  $\alpha$ -L-AFase, results in relieving the steric hindrance and exposing additional sites at which xylanase can act (Saha 2000; Kim *et al.*, 2004; Sørensen *et al.*, 2006a;b). The cooperative action of  $\alpha$ -L-AFase with xylanase was reported for  $\alpha$ -L-AFases from *Thermomonospora fusca* (Bachmann and McCarthy, 1991), *Aspergillus awamori* (Wood and McCrae, 1996), *Cytophaga*

*xylanolytica* (Renner and Breznak, 1998), *Bacillus stearothermophilus* No.236 (Kim *et al.*, 2004) and *Aureobasidium pullulans* (De Wet *et al.*, 2008). However, in all previously reported cases the amount of released sugars and hydrolysis enhancements caused by the action of the  $\alpha$ -L-AFases were less than that obtained from  $\alpha$ -L-AFase of the *Bacillus* sp NIOCCW19.

### **Saccharification of sugar cane bagasse**

Sugarcane (*Saccharum officinarum*) is a grass that is harvested for its sucrose content. After extraction of sugar from the sugarcane, the plant material that remains is termed bagasse. India produces nearly 80 million metric tonne (MMT) of bagasse (Ghosh and Ghose, 2003). Sugarcane bagasse found at sugar mills contains both relatively easy and hard to degrade materials which are appeared to be from the leaf matter and from the rind, respectively (Fox *et al.*, 1987). Bagasse has several advantages for use in ethanol production. Most importantly, unlike corn stover, bagasse is collected as part of the sugar production process, so it does not require a separate harvest. It is also physically ground as a part of the extraction process (Fox *et al.*, 1987). Furthermore, bagasse is cheap, readily available, and has high carbon content (Martín, 2002).

### **The potential sugars yield of sugar cane bagasse**

Total carbohydrates analysis showed that bagasse contains acid soluble sugars of 689.6 mg glucose equivalent/g of bagasse that represents 68.96% of the dry weight of bagasse sample. Similar results have been obtained from sugar cane bagasse where total carbohydrates accounted for 70.9 % (Pessoa *et al.*, 1997) and 64.1% (Martín *et al.*, 2006). Moreover, lignocellulosic biomass typically contains 55-75% (w/w) carbohydrates (Mosier *et al.*, 2005). The acid-insoluble material is expected to be composed of lignin and other condensable

compounds (Silverstein *et al.*, 2007). The acid –insoluble materials represented 31.04 % (w/w) of dry bagasse sample.

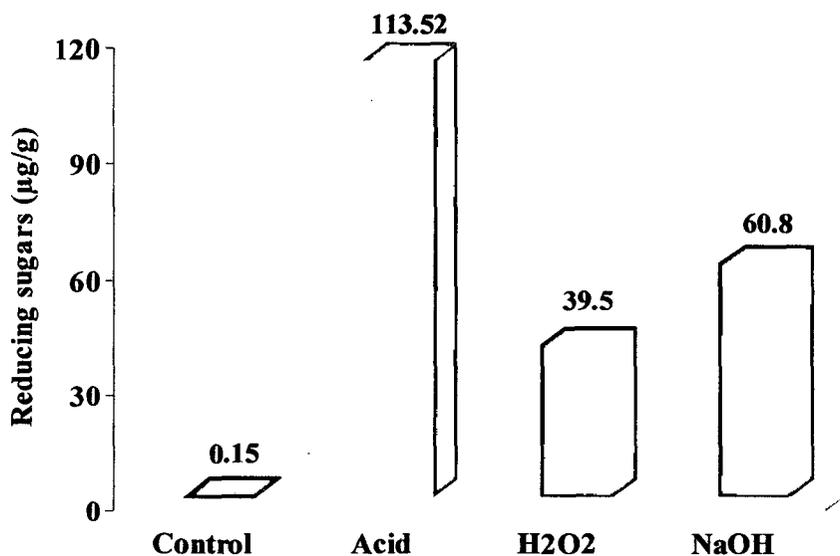
### **Chemical pretreatments:**

Pretreatment is required to alter the structure of lignocellulosic biomass and make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars. The goal is to break the lignin seal and disrupt the crystalline structure of cellulose. Pretreatment has been viewed as one of the most expensive processing steps in conversion of lignocellulosic materials to fermentable sugars. Pretreatment also has great potential for improvement of the efficiency and lowering of cost through research and development (Lee *et al.*, 1994; Kohlman *et al.*, 1995; Lynd *et al.*, 1996; Mosier *et al.*, 2003a,b). An effective pretreatment is characterized by several criteria. It avoids the need for reducing the size of biomass particles, preserves the pentose (hemicellulose) fractions, limits the formation of degradation products that inhibit growth of fermentative microorganism, minimizes energy demands and limits cost (National Research Council, 1999). These properties, along with others including low pretreatment catalyst cost or inexpensive catalyst recycle, and generation of higher-value lignin co-product form a basis of differentiation between various pretreatment options (Ladisich *et al.*, 1983; Wyman, 1995; Delgenes *et al.*, 1996; Lynd, 1996; Wyman, 1996;1999; Palmqvist and Hahn-Hagerdal, 2000; Mosier *et al.*, 2005).

### **Diluted acid pretreatment**

After treatment with 0.2 M HCl at 130°C for 30 min, the amount of sugar released from sugar cane bagasses was found to be 113.52 mg/g bagasse which

represents a yield of 16.46% of total sugar initially present in bagasse (Fig. 5.1.2). The amount of sugar released was slightly lower than that obtained in an earlier study using acid hydrolysis at 130 °C for 30 min (Kurakake *et al.*, 2005). Temperature, treatment time, and the sugar cane variety are among probable reasons for these differences in sugar yields.



**Fig. 5.1.2:** Reducing sugars obtained by the effect of chemical pretreatments on raw bagasse. Numbers in the figure represent the amount of reducing sugar obtained by each treatment.

#### **Hydrogen peroxide pretreatment:**

Peroxide pretreatment enhances enzymatic conversion through oxidative delignification and reduction of cellulose crystallinity (Gould, 1984). It increases lignin solubilization and cellulose availability and loosens the lignocellulosic matrix thus improving enzyme digestibility (Martel and Gould, 1990; Yang *et al.*, 2002; Kim *et al.*, 2001; Silverstein *et al.*, 2007). After treatment with 2% (w/v) H<sub>2</sub>O<sub>2</sub> at 121 °C and 15 psi for 90 min, the amount of sugar released from sugar cane bagasse was found to be 39.5 mg/g bagasse (Fig. 5.1.2) which represents a yield of 5.73% of total sugar initially present in

bagasse. This hydrolysis yield is lower than that obtained from cotton stalk with the same treatment (Silverstein *et al.*, 2007). This could be attributed to the structural differences between sugar cane bagasse and cotton stalk.

#### **Alkaline pretreatment:**

Alkaline pretreatment of lignoculoses is an alternative to acid pretreatment. Alkaline pretreatment increases the surface area, allowing penetration of water molecules to the inner layers of lignocellulosic biomass (Fox *et al.*, 1987). The main effect of sodium hydroxide pretreatment on lignocellulosic biomass is delignification by breaking the ester bonds cross-linking lignin and xylan thus increasing the porosity of biomass (Tarkov and Feist, 1969). This in turn increases the accessibility of hemicellulose and cellulose degrading enzymes to their substrates. After treatment with 2% (w/v) NaOH at 80°C for 1.5 h, the amount of sugar released from sugar cane bagasse was found to be 60.8 mg/g bagasse which represents a yield of 8.82% of total sugar initially present in bagasse (Fig. 5.1.2). This hydrolysis yield is lower than that obtained from maize straw (Chen *et al.*, 2008) and cotton stalk (Silverstein *et al.*, 2007) pretreated with NaOH. This could be attributed to the differences in NaOH concentration, treatment time and nature of the substrates. Moreover, the yield obtained was slightly higher than that obtained from sugar cane bagasse pretreated with 1% NaOH at 125°C for 1h (Adsul *et al.*, 2005). According to Silverstein *et al.* (2007) the effectiveness of NaOH pretreatment depends on NaOH concentration and residence time of treatment. Temperature increases the hydrolysis rate only when high concentration of NaOH up to 2% combined with longer treatment time up to 90 min is used. Moreover, Varga *et al.* (2002) reported that pretreatment of corn stover with 10% NaOH in an autoclave for 1 h resulted in

95% reduction of lignin content. However, this was associated with the degradation of hemicellulosic sugars.

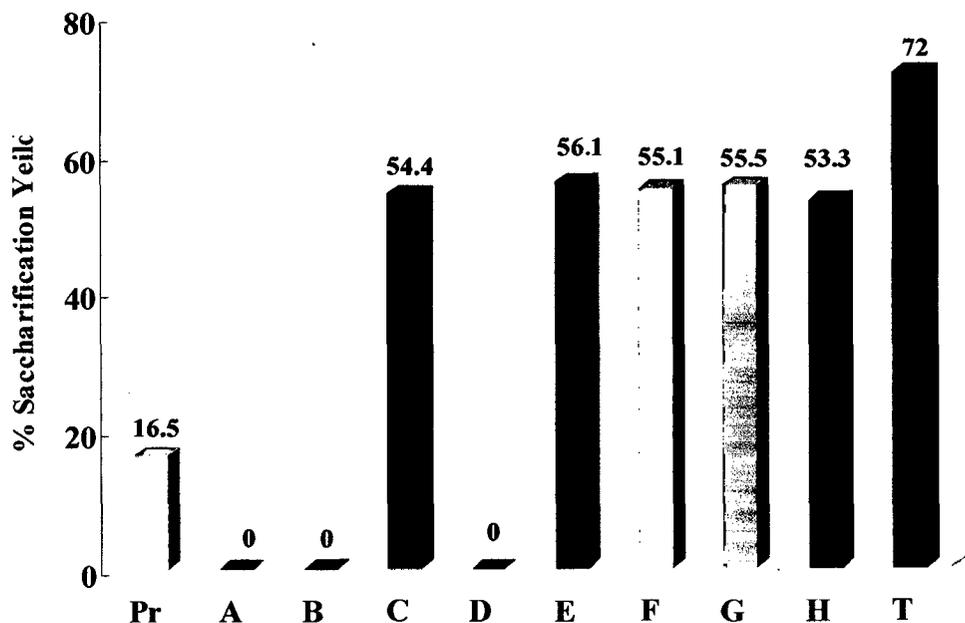
### **Enzymatic treatments of diluted acid pretreated bagasse**

The amount of reducing sugar released from diluted acid pretreated bagasse by the action of *Bacillus* sp  $\alpha$ -L-AFase and xylanase and cellulases is presented in **Table 5.1.2**. The enzymatic treatment of acid pretreated bagasse with cellulases increased the release of sugars that reach the maximum (374.85 mg/g bagasse) after 4 days. The sugar yield obtained by this treatment represents 54.358% of total sugar initially present in bagasse (**Table 5.1.2** and **Fig. 5.1.3**). However, enzymatic hydrolysis with  $\alpha$ -L-AFase and/or xylanase did not increase the sugar concentration (**Table 5.1.2**). Because no sugars were obtained when  $\alpha$ -L-AFase and/or xylanase were used this may indicate that the sugars obtained were released by the effect of acid and cellulases treatments only. This total sugar yield is actually the most important yield since it deals with all process steps from the raw bagasse to fermentable sugars. If sugars are degraded during the pretreatment step one can get high yields during enzymatic hydrolysis anyway. However, this loss of sugars will have negative impact on total yields (Chandel *et al.*, 2007). The effectiveness of this process could be influenced by different factors. The acid treatment seems to affect pentose in hemicellulose causing their destruction and conversion to furfural and furfural derivatives (Chandel *et al.*, 2007). This may explain the low yield obtained by this processes. It may also explain why  $\alpha$ -L-AFase and xylanase did not increase the sugar yield from acid pretreated bagasse because of the destruction of hemicelluloses.

**Table 5.1.2:** The amount of reducing sugars (mg/g) released by the action of enzymatic hydrolysis of chemically pretreated bagasse

Treatment	Control	Diluted HCl	Hydrogen peroxide	Sodium hydroxide
A	2.9	0	8.1	10.23
B	21.39	0	59.13	85.84
C	28.12	374.85	138.8	298.71
D	31.72	0	145.71	209.44
E	28.5	386.89	141.05	302.75
F	41.57	379.74	210	433.2
G	49.19	382.99	255.86	498.87
H	48.72	367.85	272.26	514.07

A: AFase; B: Xylanase; C: Cellulases; D: AFase and Xylanase; E: AFase and Cellulases; F: Xylanase and Cellulases; G: AFase, Xylanase and Cellulases; H: Sequential treatment with AFase and Xylanase for 24 h followed by Cellulases for 72 h.

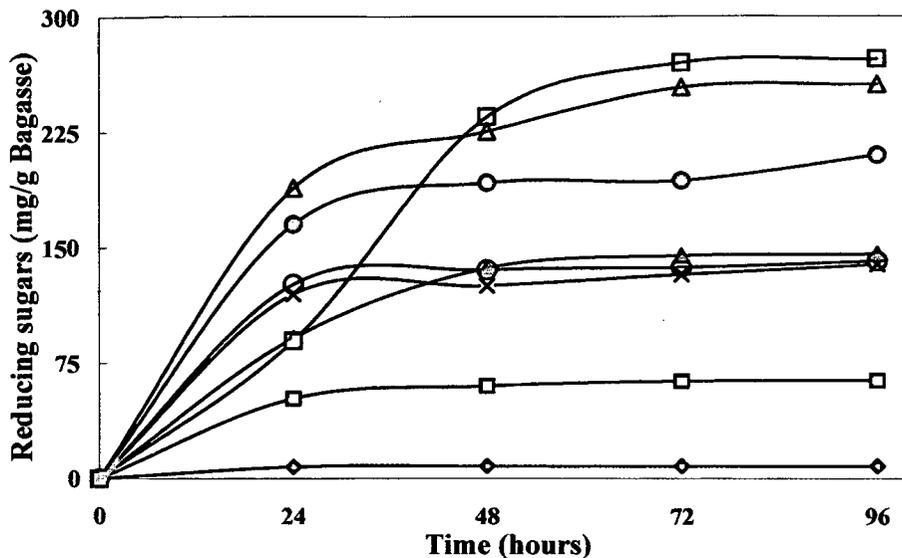


**Fig.5.1.3:** Saccharification yields obtained by enzymatic treatments of acid pretreated bagasse. Pr: Acid pretreatment; A: AFase; B: Xylanase; C: Cellulases; D: AFase and Xylanase; E: AFase and Cellulases; F: Xylanase and Cellulases; G: AFase, Xylanase and Cellulases; H: Sequential treatment with AFase and Xylanase for 24 h followed by Cellulases for 72 h; T: Overall saccharification by acid pretreatment and enzymes. Numbers in the figure represent the saccharification yield obtained by each treatment.

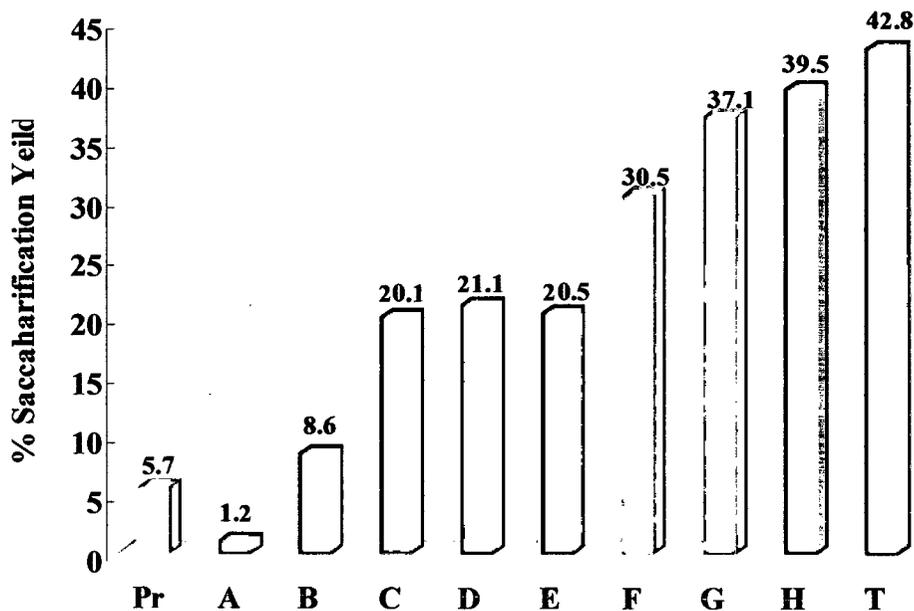
Destruction of hemicellulosic pentose is an adverse effect of acid treatment that cause low sugar yield from different agricultural residues (Chandel *et al.*, 2007). Thus, acid pretreatment of bagasse did not increase sugar yield, a vital factor for economic production of fermentable sugars for some industrial application such as bioethanol production.

### **Enzymatic treatments of H<sub>2</sub>O<sub>2</sub> pretreated bagasse**

Time course of enzymatic hydrolysis of H<sub>2</sub>O<sub>2</sub> pretreated bagasse by *Bacillus* sp  $\alpha$ -L-AFase and xylanase and cellulases is shown in **Figure 5.1.4**. The amount of reducing sugar released by the action of each enzymatic treatment is presented in **Table 5.1.2**. The action of either of  $\alpha$ -L-AFase and xylanase on H<sub>2</sub>O<sub>2</sub> pretreated bagasse characterized by increased in sugar yield that has been observed up to 24 h, thereafter sugar yield did not change significantly (**Fig. 5.1.4**). Enzymatic treatment of H<sub>2</sub>O<sub>2</sub> pretreated bagasse resulted in sugar release of 8.1, 59.13 and 138.80 (mg/g bagasse) when treated with  $\alpha$ -L-AFase, xylanase and cellulases, respectively (**Table 5.1.2**). This corresponds to a sugar yield of 1.18%, 8.57% and 20.13% of total sugars initially present in bagasse. The overall effects of hydrogen peroxide preteatment and treatment with these enzymes is shown in **Figure 5.1.5**. However, treatment with a mixture of  $\alpha$ -L-AFase and xylanase released more sugars that reach maximum value (145.71 mg/g bagasse) after 48 h. This amount of the released sugars represents 21.13 % of the total sugar initially present in bagasse (**Table 5.1.2** and **Figs. 5.1.4 & 5.1.5**). Moreover, the sugars yield was more when both enzymes were used together than the sum of yields obtained when each of the enzymes was used individually. This could be attributed to the cooperative action of  $\alpha$ -L-AFase



**Fig. 5.1.4:** Time course of the released sugars by enzymatic hydrolysis of hydrogen peroxide pretreated bagasse. ◇:AFase; □:Xylanase; x:Cellulases; Δ:AFase and xylanase; ○:AFase and cellulases; ○:xylanase and cellulases; Δ: AFase, xylanase and cellulases; □: Sequential treatment with AFase and xylanase for 24h followed by cellulases for 3 days.



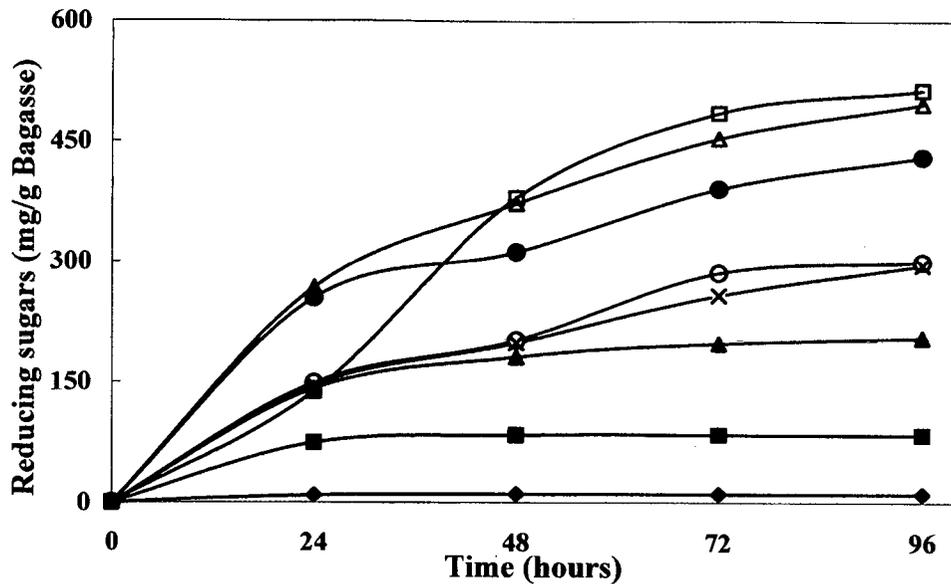
**Fig.5.1.5:** Saccharification yields obtained by enzymatic treatments of hydrogen peroxide pretreated bagasse. Pr: Acid pretreatment; A: AFase; B: Xylanase; C: Cellulases; D: AFase and Xylanase; E: AFase and Cellulases; F: Xylanase and Cellulases; G: AFase, Xylanase and Cellulases; H: Sequential treatment with AFase and Xylanase for 24 h followed by Cellulases for 72 h; T: Overall saccharification by hydrogen peroxide pretreatment and enzymes. Numbers in the figure represent the saccharification yield obtained by each treatment.

that facilitated and enhanced the catalytic activity of xylanase in degradation of hemicellulosic xylan. Interestingly, when xylanase and cellulases used for bagasse treatment at pH 5.5, 141.05 mg/g bagasse of sugar was released that represent a sugar yield of 30.45%. However, when  $\alpha$ -L-AFase and cellulases used for bagasse treatment at pH 5.5, the sugar yield obtained was 20.45% (**Table 5.1.2** and **Figs. 5.1.4 & 5.1.5**). This may indicate that xylanase enhanced cellulases activity which is evidenced by an increase in sugar yield of 10.32% than that obtained when cellulases were used alone. However, no significant increase in sugar yield was observed when  $\alpha$ -L-AFase and cellulases were used together. This is because  $\alpha$ -L-AFase acts on arabinose branches present in xylan and further degradation with xylanase is required to expose cellulose for cellulases action. The treatment of H<sub>2</sub>O<sub>2</sub> pretreated bagasse with mixture of  $\alpha$ -L-AFase, xylanase and cellulase resulted in overall saccharification yield of 37.1% of total sugar initially present in bagasse (**Table 5.1.2** and **Fig. 5.1.5**). However, sequential treatment of H<sub>2</sub>O<sub>2</sub> pretreated bagasse first with  $\alpha$ -L-AFase- xylanase mixture and then with cellulases slightly increases the overall yield of saccharification to 41.3% of total sugar initially present in bagasse (**Fig. 5.1.5**). The overall yield of the saccharification of sugar cane bagasse was higher than that obtained in previous study (Adsul *et al.*, 2005). Many factors could contribute to the higher yield obtained in this study. These may include sugar cane variety, particle size, treatment temperature and treatment time and finally efficiency of the enzymes used. The overall saccharification yield obtained using H<sub>2</sub>O<sub>2</sub> pretreatment and enzymatic treatment was lower than the yields obtained using diluted acid or alkaline pretreatments followed by enzymatic treatment. Even though, H<sub>2</sub>O<sub>2</sub>

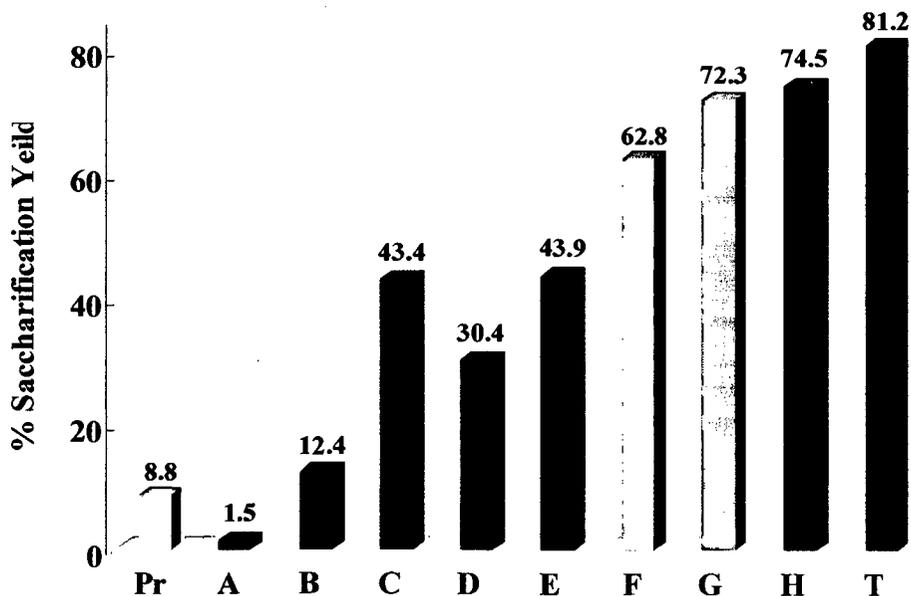
pretreatment might loosen the bagasse matrix and resulted in better enzymes access to hemicellulosic fraction of bagasse. However, it might be unable to completely unwind the crystalline structure of cellulose fraction of bagasse. This could be the reason for low yield as not all cellulose was degraded to sugar monomers.

### **Enzymatic treatments of alkaline pretreated bagasse**

Time course of enzymatic hydrolysis of NaOH pretreated bagasse by either of  $\alpha$ -L-AFase, xylanase and cellulase is shown in **Figure 5.1.6**. The amount of reducing sugar released by the action of each enzymatic treatment is presented in **Table 5.1.2**. Enzymatic treatment of NaOH pretreated bagasse resulted in the release of 10.23, 85.85, and 298.71 mg sugar/g bagasse when treated with  $\alpha$ -L-AFase, xylanase and cellulases, respectively (**Table 5.1.2** and **Fig. 5.1.6**). Thus, yields of 1.48%, 12.45%, and 43.35% of initial sugar present in bagasse were obtained by the enzymatic treatment with  $\alpha$ -L-AFase, xylanase and cellulases, respectively (**Fig. 5.1.6** and **Fig. 5.1.7**). Furthermore,  $\alpha$ -L-AFase and xylanase mixture released 209.4 mg/g bagasse that represent 30.37 % sugar yield which is higher than the sum of yields obtained when each enzyme treatment was carried out individually (**Table 5.1.2** and **Figs. 5.1.6 & 5.1.7**). The significant increase in sugar yields may reflect the synergistic acts of these enzymes (**Table 5.1.2** and **Fig. 5.1.7**). The saccharification yield obtained may also reflect the amount of hemicelluloses present in bagasse. This was suggested because hemicelluloses were reported to be between 20-35% of carbohydrates present in bagasse (Kuhad and Singh, 1993, Saha, 2003; Martín *et al.*, 2006; Chandel *et al.*, 2007). It was observed that xylanase enhanced the catalytic activity of cellulases in degradation of NaOH pretreated bagasse.



**Fig. 5.1.6:** Time course and released sugars by enzymatic hydrolysis of sodium hydroxide pretreated bagasse. ◆:AFase; ■:Xylanase; ×:Cellulases; ▲:AFase and xylanase; ○:AFase and cellulases; ●:xylanase,cellulases; △: AFase, xylanase and cellulases; □: Sequential treatment with AFase and xylanase for 24h followed by cellulose for 3 days.



**Fig. 5.1.7:** Saccharification yields obtained by enzymatic treatments of alkaline pretreated bagasse. Pr: Acid pretreatment; A: AFase; B: Xylanase; C: Cellulases; D: AFase and Xylanase; E: AFase and Cellulases; F: Xylanase and Cellulases; G: AFase, Xylanase and Cellulases; H: Sequential treatment with AFase and Xylanase for 24 h followed by Cellulases for 72 h; T: Overall saccharification by alkaline pretreatment and enzymes. Numbers in the figure

It was found that 62.82% sugar yield was obtained when both enzymes were used together for treatment. Furthermore, the yield obtained was higher than the sum of yields obtained when each enzyme treatment was carried out individually (Fig. 5.1.7). The results showed no significant increase in saccharification yield when  $\alpha$ -L-AFase and cellulases were used together for the treatment. The enzymatic treatment of NaOH pretreated bagasse with mixture of  $\alpha$ -L-AFase, xylanase and cellulase resulted in overall enzymatic saccharification yield of 72.34% of total sugar initially present in bagasse (Table 5.1.2 and Fig. 5.1.7). Moreover, no significant increase in the overall yield of enzymatic treatment was observed with sequential treatment first with  $\alpha$ -L-AFase- xylanase mixture and then with cellulases. The overall saccharification yield obtained by alkaline (NaOH) and enzymatic treatment was 81.2% of original sugar content initially present in bagasse (Fig. 5.1.7). The saccharification yield obtained was higher than that obtained in previous studies from sugar cane bagasse (Kurakake *et al.*, 2001;2005; Adsul *et al.*, 2005; Martín *et al.*, 2006), sunflower hulls (Sharma *et al.*, 2004) and rice hulls (Saha *et al.*, 2005). However, it was slightly lower than that obtained from maize straw (Chen *et al.*, 2008) and corn husks (Kurakake *et al.*, 2001).

The fact that  $\alpha$ -L-AFase and xylanase worked cooperatively with cellulases at low pH (5.5) was interesting. This is because the optimum activity of  $\alpha$ -L-AFase and xylanase are at pH 8 and pH 9, respectively. This could be attributed to the presence of the enzymes substrates which help in more stabilization of these enzymes (Volkin and Klivanov, 1989; Asther and Meunier, 1993; Gomes *et al.*, 2000; Sørensen *et al.*, 2003; Wilson and Walker, 2005; Sørensen *et al.*, 2006a; Silverstein *et al.*, 2007). Moreover,  $\alpha$ -L-AFase

showed 73.1% and 76.04% relative activity and stability for 24 h at the pH 5.0. Furthermore, xylanase showed 64.1% and 51.13% relative activity and stability for 24 h at the pH 5.5.

Comparing the overall saccharification yields obtained by the chemical and enzymatic treatments revealed the superiority of NaOH pretreatment and the enzymatic mixture  $\alpha$ -L-AFase, xylanase and cellulases for saccharification of sugar cane bagasse. The NaOH pretreatment of bagasse provided the highest biomass conversion to reducing sugars during subsequent enzymatic hydrolysis with a mixture of  $\alpha$ -L-AFase, xylanase and cellulases. The synergistic effect of  $\alpha$ -L-AFase and xylanase was further confirmed by their action on hemicelluloses released from NaOH and H<sub>2</sub>O<sub>2</sub> pretreated bagasse. Nevertheless,  $\alpha$ -L-AFase showed minor effect in releasing reducing sugars from pretreated bagasse. However,  $\alpha$ -L-AFase action facilitates markedly the action of xylanase in releasing increased amounts of reducing sugars.

According to Ghosh and Ghose, (2003) utilization of such enzymatic saccharification of lignocelluloses has several advantages for bioethanol production. These include higher saccharification yields, no production of inhibitors, can be coupled with fermentation in one step, less effluents and environment friendly (Ghosh and Ghose, 2003).

Taken together, these results reinforced that the cooperative action of  $\alpha$ -L-AFase and xylanase is essential for complete hydrolysis of arabinoxylans present in hemicelluloses, especially those containing large amount of arabinose side chains.

Comparative Evaluation of Catabolite Repression in  
Solid-State Fermentation and Submerged Fermentation for  
 $\alpha$ -L-arabinofuranosidase and xylanase Biosynthesis by *Bacillus* sp

### 5.2.1 INTRODUCTION

Carbon catabolite repression is a regulatory mechanism that represses the expression of genes involved in utilization of alternative carbon sources in the presence of energetically more favorable one such as D-glucose and D-fructose (Hueck and Hillen, 1995; Brückner and Titgemeyer, 2002). Many genes encoding for carbon catabolic enzymes in *Bacillus subtilis* are transcriptionally controlled by catabolite repression (CR) (Fisher and Sonenshein, 1990; Saier, 1991; Chambliss, 1993; Mota *et al.*, 2001; Brückner and Titgemeyer, 2002).  $\alpha$ -L-arabinofuranosidase ( $\alpha$ -L-AFase) and xylanase are hemicelluloses degrading enzymes produced by a range of lignocellulose-degrading microorganisms (Beldman *et al.*, 1997; Saha, 2000; 2003). In most microorganisms production of these enzymes is inducible and under control of carbon catabolite repression (Saha, 2003; Gueimonde *et al.*, 2007). Many studies reported the use of submerged fermentation (SmF) for the production of  $\alpha$ -L-AFase or xylanase but not both the enzymes together (Weinstein and Albersheim, 1979; Morales *et al.*, 1995; Gilead and Shahom, 1995; Debeche *et al.*, 2000; Kosugi *et al.*, 2002). However, reports on the biosynthesis of these two enzymes together are scanty (Bachmann and McCarthy, 1991). In addition, it is not yet clear whether production of these two enzymes is under the control of the same type of regulation mechanism. Nevertheless,  $\alpha$ -L-AFase and

xylanase are co-induced by arabinose, xylose or by polymers containing these sugars in SmF (Gat *et al.*, 1994; Gilead and Shahom, 1995; Khanderparker and Ghosle, 2006; Khanderparker *et al.*, 2008). However, the effect of catabolite repression on their biosynthesis in solid state fermentation (SSF) remains uncertain. Furthermore, studies indicated that the biosynthesis of these enzymes was repressed in the presence of glucose in SmF medium (Shoham *et al.*, 1992; Van Laere *et al.*, 1999; Carvallo *et al.*, 2003; Bocchini *et al.*, 2008). Thus, the catabolite repression of biosynthesis of bacterial  $\alpha$ -L-AFase and xylanase poses serious problem of economy in SmF. Because under SmF, catabolite repression causes severe reduction in production of these enzymes (Shoham *et al.*, 1992; Bocchini *et al.*, 2008). Conversely, SSF has the ability to minimize the effect of catabolite repression on the biosynthesis of some extracellular enzymes by bacteria (Ramesh and Lonsane 1991; Babu and Satyanarayana, 1995) and fungi (Te Biesebeke *et al.*, 2005; Nandakumar *et al.*, 1999). In view of this, it was thought worthwhile to study catabolite repression of glucose on  $\alpha$ -L-AFase and xylanase biosynthesis by *Bacillus* sp NIOCCW19 when grown under SSF and SmF conditions. An attempt was, therefore, made to explain the differences in the yields of  $\alpha$ -L-AFase, xylanase and biomass.

## 5.2.2 MATERIALS and METHODS

### 5.2.2.1 Inoculum preparation

Bacterial inoculum was prepared following the method described by Ramesh and Lonsane (1991). To prepare the inoculum, *Bacillus* sp NIOCCW19 was grown on ZMA plates for 24 h. A loop full of grown bacteria on ZMA plate was used to inoculate 0.2 % glucose medium. For preparation of glucose medium,

the MBSM2 (**Table 4.1.1, Section 1 of Chapter 4**) was sterilized at 121°C for 15 min and its pH was adjusted to pH, 8.00 with 1N NaOH. Concentrated glucose solution was sterilized separately and added to the sterilized optimized medium to give a final concentration of 0.2%. The growth of *Bacillus* sp NIOCCW19 in this medium was monitored by measuring growth O.D. at 600 nm. The cells of *Bacillus* sp were grown up to the stationary growth phase. They were then separated by centrifugation at 10,000 RPM for 20 min at 4°C. The bacterial cells were suspended in sterile saline and used as inoculum. Viable bacterial cells present in the suspension were determined by serial dilution and plating on ZMA plates. The plates were incubated at room temperature for 48 h and the grown bacterial colonies were counted. The plate count was  $8 \times 10^8$  bacterial cell ml<sup>-1</sup> of bacterial suspension.

#### **5.2.2.2 $\alpha$ -L-AFase and xylanase production by submerged fermentation**

SmF was performed in 250 ml Erlenmeyer flasks, containing 2 % commercial wheat bran and 50 ml of SmF-MBSM2 (**Table 4.1.1**). The flasks sterilized as above and cooled to room temperature. Concentrated glucose solutions were sterilized separately and added to each of these flasks to give final glucose concentrations from 5 to 150 mg ml<sup>-1</sup> in a 50 ml final volume. The flasks were inoculated with 3 ml of the bacterial cell suspension prepared as above and incubated at 40°C on rotary shaker at 100 RPM for the desired period.

#### **5.2.2.3 $\alpha$ -L-AFase and xylanase production by solid state fermentation**

SSF was conducted in 250 ml Erlenmeyer flasks. The Flasks contained 4.5 g of commercial wheat bran moisturized with the SSF-MBSM2 (**Table 4.1.1**) in a ratio of 1: 3 (substrate: SSF-MBSM2). The flasks sterilized as above and cooled

to room temperature. Concentrated glucose solutions were added to each of these flasks to give glucose concentrations from 5.0 to 150 mg g<sup>-1</sup> substrate. Care was taken to adjust the final moisture level to 1:3 (w/v). The flasks were then inoculated with 1 ml of the bacterial cell suspension and incubated at 40°C under static conditions for the desired period. Before incubation the flasks were gently tapped intermittently to mix the contents.

#### **5.2.2.4 Analytical methods:**

Periodically, the whole content of SSF medium flasks and samples of SmF medium were drawn. The content of SSF flask was treated with 50mM Na-phosphate buffer pH 8.0 on rotary shaker at 200 RPM for 1 h. The treated content of SSF flask and SmF samples were centrifuged at 10,000 RPM for 10 min. The precipitated pellets were washed several time with 50 mM Na-phosphate buffer pH 8 and used to estimate bacterial protein biomass. The protein content of the cell was estimated following the method described in Chapter 4 section 1. The clarified supernatants were used for enzymes assays and residual glucose estimation.

$\alpha$ -L-arabinofuranosidase activity was determined as described in Chapter 4 section 1. Xylanase activity was measured as described in Chapter 2. The residual glucose in the media was determined using the glucose oxidase-peroxidase method (Bergmeyer and Bernt, 1974) following de Souza *et al.*, (2001).

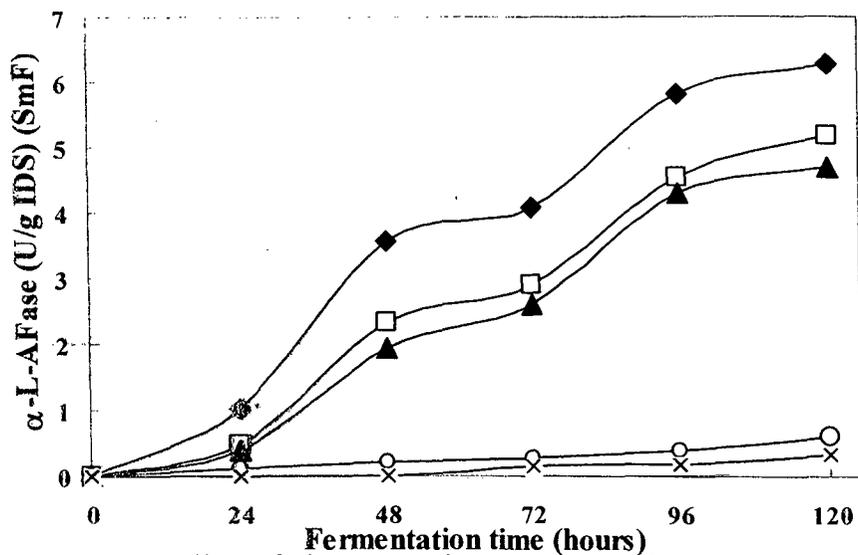
Comparing enzymes titers and bacterial biomass production in SSF and SmF was done as described in section 1 of Chapter 4. Each experimental value represented the average of two independent determinations. The standard deviations SD $\pm$  of these reading were less than 8% of the calculated mean.

### 5.2.3 RESULTS and DISCUSSION

Gram-positive bacteria from the *Bacillus* group can use various carbohydrates as a single source of carbon and energy (Stulke and Hillen, 2000). Using extracellular carbohydrases, bacilli degrade several polysaccharides that are widely distributed in plant cell walls (Rodionov *et al.*, 2001). Hemicellulose polysaccharides are degraded by variety of extracellular xylanolytic enzymes such as  $\alpha$ -L-AFase and xylanase. The induction of the *Bacillus subtilis* pentose catabolic operons, as well as global carbon catabolite repression, is mediated by several transcriptional repressors. The utilization of arabinose and xylose is controlled by AraR and XylR, respectively. The AraR and XylR control the biosynthesis of  $\alpha$ -L-AFase and xylanase, respectively. The catabolite repressor protein CcpA represses transcription of catabolic genes by binding to a palindromic sequence called CRE (catabolite responsive element) (Hueck and Hillen, 1995; Rodionov *et al.*, 2001; Brückner and Titgemeyer, 2002). Repression by glucose is common for catabolic extracellular enzymes produced in SmF (Toda, 1981; Biely, 1982; Balakrishnan *et al.*, 1997; Abdel-Fattaha *et al.*, 2005; Bocchini *et al.*, 2008).

#### **Catabolite repression of $\alpha$ -L-AFase and xylanase in SmF**

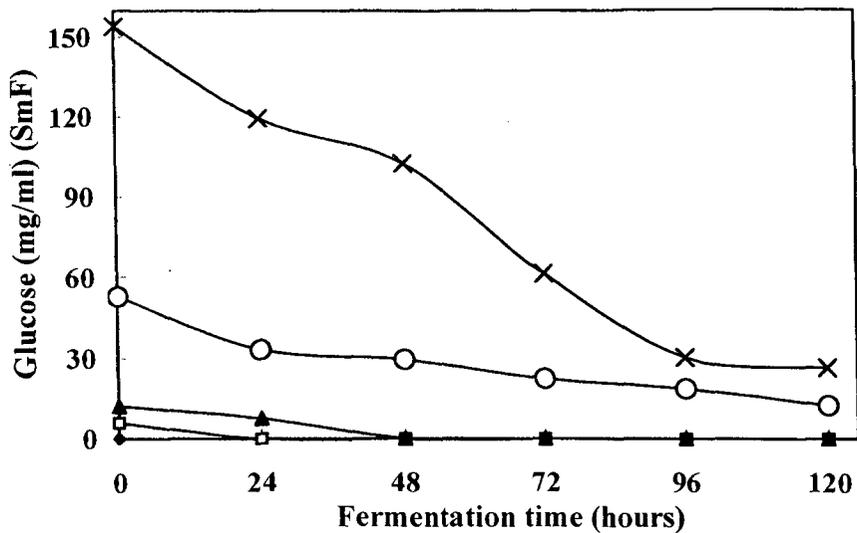
The effect of glucose on the production of  $\alpha$ -L-AFase by *Bacillus* sp NIOCCW19 grown in SmF is presented in **Figure 5.2.1**. The biosynthesis of  $\alpha$ -L-AFase by the bacterium in SmF was highly influenced by the presence of glucose in the growth medium. The  $\alpha$ -L-AFase production was lower, at all glucose concentrations tested than that in glucose free control medium. The presence of low (5 and 10 mg ml<sup>-1</sup>) glucose concentrations in wheat bran SmF based medium caused moderate effect on  $\alpha$ -L-AFase biosynthesis (**Fig. 5.2.1**).



**Fig. 5.2.1:** The effect of glucose on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in submerged fermentation (SmF). (◆) 0mg, (□) 5 mg, (▲) 10 mg, (○) 50 mg, (×) 150 mg.

However,  $\alpha$ -L-AFase production was reduced by 17.4% and 24.92% in SmF medium when supplemented with 5 and 10 mg ml<sup>-1</sup> of glucose, respectively. In SmF medium with high glucose concentrations (50 and 150 mg ml<sup>-1</sup>), severe reduction in  $\alpha$ -L-AFase biosynthesis was observed. By the end of fermentation period (120 h), the  $\alpha$ -L-AFase biosynthesis was reduced by more than 90 % in SmF medium containing glucose at concentrations of 50 and 150 mg ml<sup>-1</sup> (Fig. 5.2.1). Meanwhile, gradual decrease in glucose concentration was observed in these media up to 96h of fermentation period. Thereafter, no significant change in glucose concentrations was observed in this media (Fig.5.2.2).

The effect of glucose on the biosynthesis of  $\alpha$ -L-AFase in SmF depended on the enzyme and microorganism. In *Bifidobacterium adolescentis* DSM 20083,  $\alpha$ -L-AFase not produced in SmF medium contained either of glucose, arabinose and arabinoxylan polymer, whereas,  $\alpha$ -L-AFase produced in the medium containing arabino-xylooligosaccharides (Van Laere *et al.*, 1999). The production of  $\alpha$ -L-AFase by *Bifidobacterium longum* was inhibited in

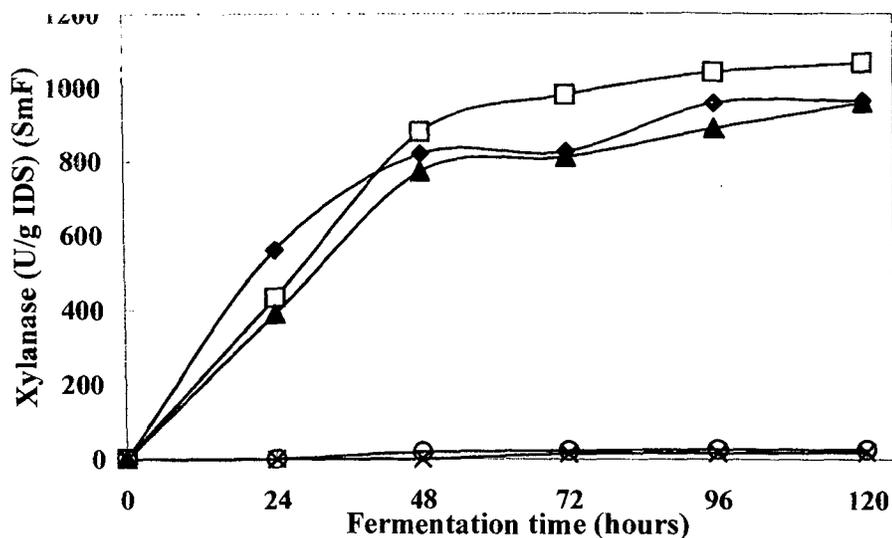


**Fig. 5.2.2:** Residual glucose level in submerged fermentation. (◆) 0mg, (□) 5 mg, (▲)10 mg, (○) 50 mg, (×) 150 mg.

SmF medium containing glucose (Gueimonde *et al.*, 2007). In contrast, growing *Streptomyces diastaticus* in wheat bran SmF based medium supplemented with 5g/L glucose did not affect the production of  $\alpha$ -L-AFase (Tajana *et al.*, 1992). In some fungi, production of  $\alpha$ -L-AFase in SmF media was repressed in the presence of glucose. These included, *Aspergillus niger* (Ruijter *et al.*, 1997); *Aspergillus nidulans* (Gielkens *et al.*, 1999); *Penicillium purpurogenum* (De Ioannes *et al.*, 2000; Carvallo *et al.*, 2003); and *Prevotella ruminicola* B<sub>14</sub> (Gasparic *et al.*, 1995b). However, in *Aspergillus terreus* CECT 2663 delay in  $\alpha$ -L-AFases production was observed for 12 h in SmF medium containing arabitol and glucose (Le Clinche *et al.*, 1997). In *Corticium rolfsii* presence of glucose reduces  $\alpha$ -L-AFase production but did not cause complete inhibition (Kaji and Yoshihira, 1969). In contrast, in *Trichoderma reesei* RUT C-30 addition of glucose did not repress  $\alpha$ -L-AFase production (Kristufek *et al.*, 1994). According to the authors, that was not a specific feature of the strain used (RUT C-30), but has also been observed with other *T. reesei* strains (Kristufek *et al.*, 1994). Furthermore, there are some reports indicated that

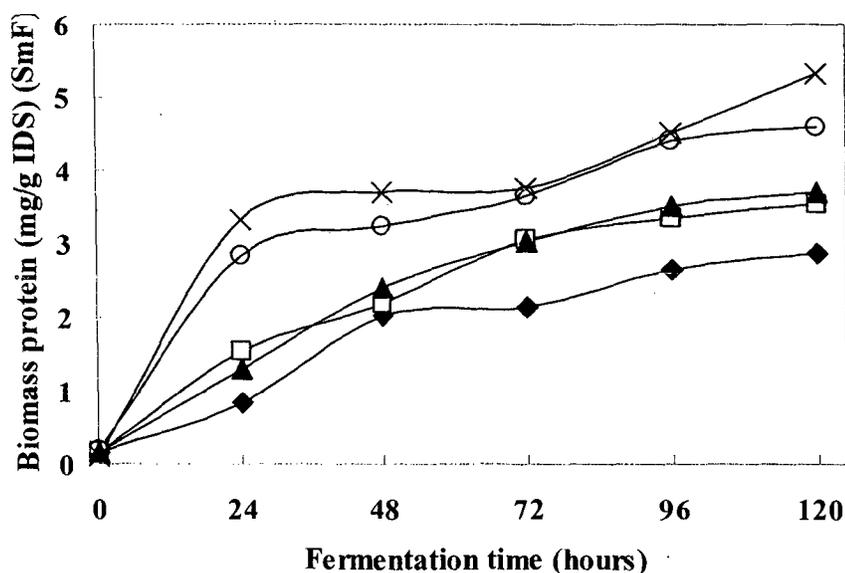
glucose has stimulatory effect on  $\alpha$ -L-AFase production in SmF media. The glucose SmF based media was used for production of  $\alpha$ -L-AFase by *Rhodotorula flava* (Uesaka *et al.*, 1978) and *Rhodotorula glutinis* (Martínez *et al.*, 2006). In addition, *Penicillium chrysogenum* grown under SmF on mixed carbon sources of glucose and sugar beet pulp efficiently produce  $\alpha$ -L-AFase (Sakamoto and Kawasaki, 2003). Similar observation was recorded for production of extracellular pectinases from *Aspergillus* sp in SmF growth medium (Aguilar *et al.*, 1987).

The effect of glucose on the production of xylanase by *Bacillus* sp NIOCCW19 grown in SmF is presented in **Figure 5.2.3**. Xylanase production was not reduced in the presence of glucose at low concentrations (5 mg ml<sup>-1</sup> and 10 mg ml<sup>-1</sup>) in SmF medium. As mentioned above, glucose was depleted by 24 h and 48 h in SmF based medium supplemented initially by 5 and 10 mg ml<sup>-1</sup> glucose, respectively (**Fig. 5.2.2**). Interestingly, presence of glucose at concentration of 5 mg ml<sup>-1</sup> in SmF medium enhanced xylanase production by 10.65%. Furthermore, no significant reduction in xylanase production was observed in the SmF medium with glucose concentration of 10 mg ml<sup>-1</sup>. However, severe reduction in xylanase production was observed in SmF media with high glucose concentrations. The xylanase production was reduced by more than 90% in SmF medium containing glucose at concentrations of 50 and 150 mg ml<sup>-1</sup> (**Fig. 5.2.3**). In *Clostridium thermolacticum* (Brodel *et al.*, 1990), *Bacillus thermoalkalophilus* (Rajaram and Varma, 1990), and *Bacillus stearothermophilus* strain T 6 (Shoham *et al.*, 1992) the synthesis of xylanase is catabolite-repressed by readily metabolizable substrates such as glucose.



**Fig. 5.2.3:** The effect of glucose on the production of xylanase by *Bacillus* sp grown in submerged fermentation (SmF). (◆) 0mg, (□) 5 mg, (▲) 10 mg, (○) 50 mg, (×)150 mg.

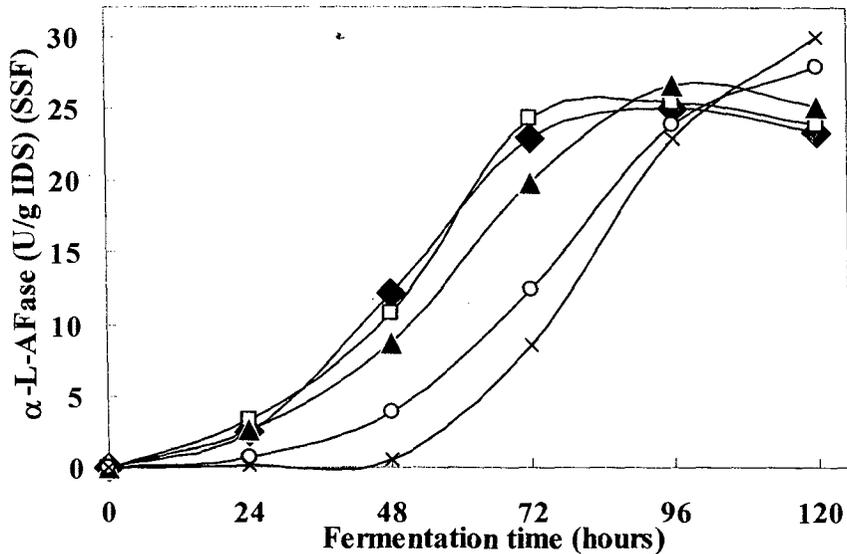
The repressive effect of glucose on xylanase production by *Bacillus circulans* D1 grown under SmF, increased with the increase in glucose concentration (Bocchini *et al.*, 2008). Bacterial biomass production was higher in glucose containing SmF media as compared to that in glucose free controlled SmF medium (Fig. 5.2.4). It was observed in glucose containing SmF medium that, biomass production increased with the increase in glucose concentration present in these medium.



**Fig. 5.2.4:** Bacterial biomass production by *Bacillus* sp grown in submerged fermentation (SmF) in the presence of different concentration of glucose. (◆) 0 mg, (□) 5 mg, (▲) 10 mg, (○) 50 mg, (×) 150 mg.

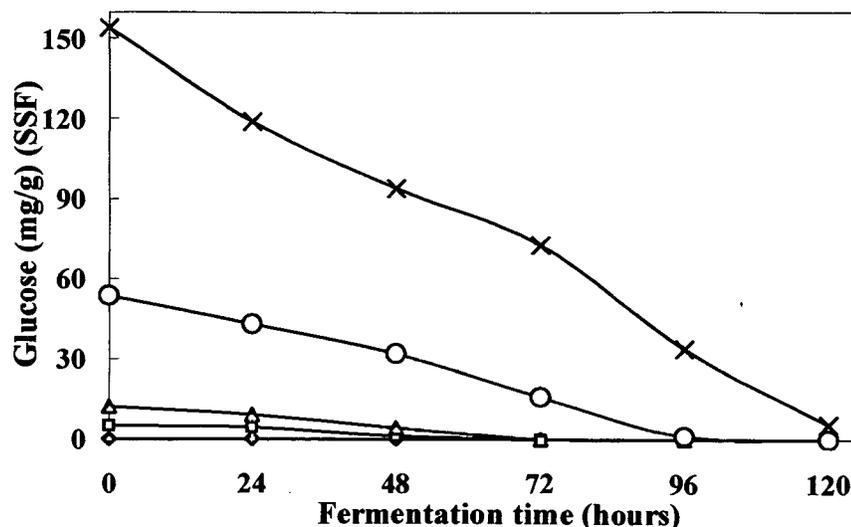
### Catabolite repression of $\alpha$ -L-AFase and xylanase in SSF

The effect of glucose on the production of  $\alpha$ -L-AFase in SSF is presented in **Figure 5.2.5**. The  $\alpha$ -L-AFase activity was detected around 24 h in SSF medium with low ( $5 \text{ mg g}^{-1}$  and  $10 \text{ mg g}^{-1}$ ) glucose concentrations as well as in glucose free controlled medium. However, slightly slower rate of  $\alpha$ -L-AFase synthesis up to 48 h was observed in low glucose SSF media as compared to those in glucose free controlled SSF medium (**Fig. 5.2.5**). Meanwhile, glucose depletion was observed after 48 h and 72 h of fermentation period in SSF based medium supplemented initially with 5 and  $10 \text{ mg ml}^{-1}$  glucose, respectively (**Fig. 5.2.6**).



**Fig. 5.2.5:** The effect of glucose on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in solid state fermentation (SSF). (◆) 0mg, (□) 5 mg, (▲) 10 mg, (○) 50 mg, (×)150 mg.

Moreover, at 120 h of fermentation period,  $\alpha$ -L-AFase production was slightly higher in these media. The results showed that at 120 h an increase of 8.18% in  $\alpha$ -L-AFase production was observed in SSF medium containing glucose at  $10 \text{ mg g}^{-1}$  compared to that observed in glucose free SSF medium.

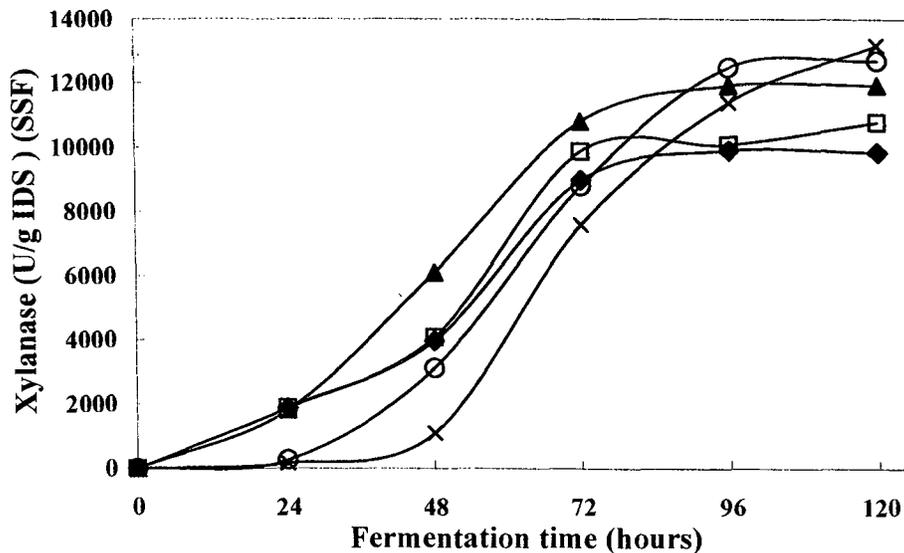


**Fig. 5.2.6:** Residual glucose level in solid-state fermentation. (◇) 0mg, (□) 5 mg, (Δ) 10 mg, (○) 50 mg, (×) 150 mg.

The presence of glucose in high concentration strongly influenced the  $\alpha$ -L-AFase biosynthesis. It has resulted in a delay in  $\alpha$ -L-AFase biosynthesis. This effect was more severe in SSF medium supplemented with 50 mg and 150 mg glucose which delayed the enzyme biosynthesis for 24 h and 48 h, respectively (Fig. 5.2.5). Nevertheless, residual glucose concentrations were still high (more than 90 mg g<sup>-1</sup> and 30 mg g<sup>-1</sup>) in these media (Fig. 5.2.6). However, the  $\alpha$ -L-AFase production in SSF medium with high glucose concentrations was increased gradually after 48h. Interestingly, compared to glucose free SSF medium at 120 h,  $\alpha$ -L-AFase production increased by 19.86% and 28.93% in SSF medium with glucose at 50 mg g<sup>-1</sup> and 150 mg g<sup>-1</sup>, respectively.

The effect of glucose on the production of xylanase in solid state fermentation (SSF) is presented in Figure 5.2.7. The presence of low glucose concentrations in SSF medium did not reduce xylanase production or delay the synthesis of the enzyme. Instead, high rate of xylanase synthesis and enzyme accumulation was

observed after 48 h in SSF medium with low glucose concentrations. Furthermore, compare to glucose free SSF medium, xylanase production at 120 h was increased by 9.51% and 21.15% in SSF media containing glucose at 5 mg g<sup>-1</sup> and 10 mg g<sup>-1</sup>, respectively (Fig. 5.2.7).



**Fig. 5.2.7:** The effect of glucose on the production of  $\alpha$ -L-AFase by *Bacillus* sp grown in solid state fermentation (SSF). (◆) 0mg, (□) 5 mg, (▲) 10 mg, (○) 50 mg, (×)150 mg.

The presence of glucose in high concentration (50 and 150 mg g<sup>-1</sup>) resulted in a delay in xylanase production (Fig.5.27). Nevertheless, the residual glucose concentration at 48 h was more than 90 mg g<sup>-1</sup> and 30 mg g<sup>-1</sup> in SSF medium that was initially supplemented with 50 mg g<sup>-1</sup> and 150 mg g<sup>-1</sup> of glucose, respectively (Fig. 5.2.6). However, a gradual increase in xylanase production and glucose consumption was observed after 48 h and up to 72 h of fermentation period in these media. At this period (72 h) glucose concentration was still high in SSF media that were initially supplemented with 50 mg g<sup>-1</sup> and 150 mg g<sup>-1</sup> (Fig. 5.2.6). Thereafter, a significant increase in xylanase production was observed in SSF medium that was initially supplemented with 50 mg g<sup>-1</sup> up to the end of fermentation period. In SSF medium that was initially

supplemented with 150 mg g<sup>-1</sup>, however, enzyme production increased significantly at 96h and up to the end of fermentation period. Interestingly, as compared to glucose free SSF medium at 120 h, xylanase production increased by 28.75% and 33.57% in SSF media with glucose at 50 mg g<sup>-1</sup> and 150 mg g<sup>-1</sup>, respectively (Fig. 5.27).

The production of bacterial biomass was higher in glucose containing SSF medium as compared to that in glucose free controlled SSF medium. Furthermore, bacterial biomass production was increased with the increase in glucose concentration present in this medium (Fig. 5.2.8).

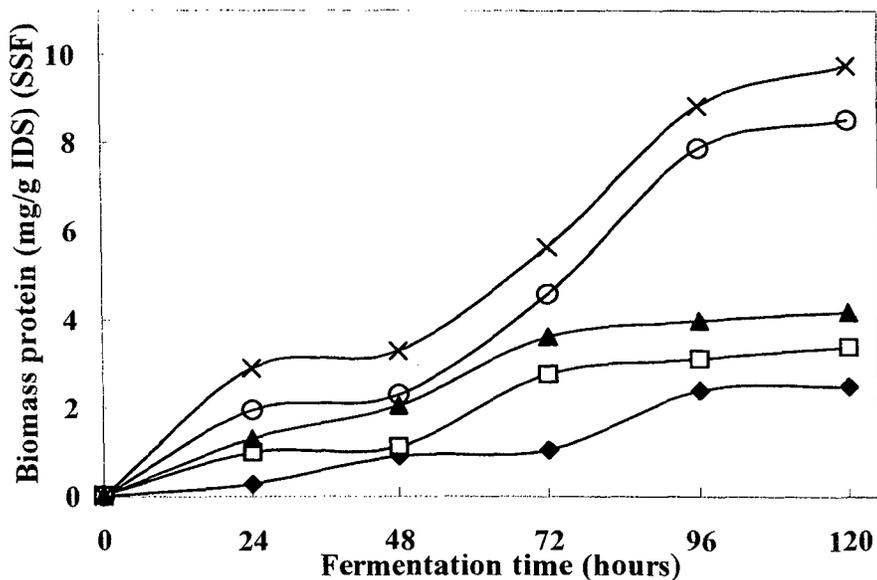


Fig. 5.2.8: Bacterial biomass production by *Bacillus* sp grown in solid-state fermentation in the presence of different concentration of glucose. (◆) 0mg, (◻) 5 mg, (▲) 10 mg, (○) 50 mg, (×)150 mg.

#### $\alpha$ -L-AFase vs. xylanase

The biosynthesis of  $\alpha$ -L-AFase seems to be more effected by the presence of low concentration of glucose when compared to xylanase in SmF. This was suggested because moderate reduction in  $\alpha$ -L-AFase production was observed compare to no effect or slight increase in xylanase production in wheat bran

based-SmF media (**Fig. 5.2.1 and Fig. 5.2.3**). However, at high glucose concentration, production of both the enzymes ( $\alpha$ -L-AFase and xylanase) was severely inhibited in wheat bran based-SmF medium (**Fig. 5.2.1 and Fig. 5.2.3**). Similarly, in *Butyrivibrio fibrisolvens* GS113 no  $\alpha$ -L-AFase or xylanase was produced in SmF medium containing glucose (Hespell and O'Bryan, 1992). In *Butyrivibrio fibrisolvens* H17c high level of xylanase produced in SmF medium containing xylan and glucose whereas production of  $\alpha$ -L-AFase was repressed in this medium (Hespell and Cotta, 1995). Production of  $\alpha$ -L-AFase and xylanase by *Thermotoga thermarum* in SmF medium was inhibited in the presence of glucose (Antranikian, 1996). In *Prevotella ruminicola* B<sub>14</sub> reduced level of  $\alpha$ -L-AFase and xylanase produced in the presence of glucose (Gasparic *et al.*, 1995b). In *Trichoderma reesei* strain QM9414 the expression of the genes responsible for  $\alpha$ -L-AFase and xylanase activities was not detected in the presence of glucose but derepressed expression occurred after glucose depletion (Margolles-Clark *et al.*, 1996).

The biosynthesis of  $\alpha$ -L-AFase and xylanase was increased with the increase in glucose concentration in wheat bran based-SSF medium (**Fig. 5.2.5 and Fig. 5.2.7**). Low concentrations of glucose have stimulatory effect on  $\alpha$ -L-AFase and xylanase production. However, biosynthesis of both the enzymes ( $\alpha$ -L-AFase and xylanase) was delayed in wheat bran based-SSF medium supplemented with high glucose concentrations (**Fig. 5.2.5 and Fig. 5.2.7**). Although, presence of high glucose concentration caused a delay in enzymes biosynthesis, it was interesting to note that the  $\alpha$ -L-AFase and xylanase production increased significantly with the increase in glucose concentration (**Fig. 5.2.5 and Fig. 5.2.7**). The delay in  $\alpha$ -L-AFase and xylanase biosynthesis

in the presence of high concentration of glucose might be accompanied with other processes that are believed to boost the level of extracellular enzyme activity (Kelly and Hynes, 1981; Ruijter and Visser, 1997). These processes included the slow release of the inducers molecules and the possibility of the culture converting the inducers to their non-metabolizable derivative (Kelly and Hynes, 1981; Kulkarni *et al.*, 1999; Ruijter and Visser, 1997).

### **Solid state fermentation vs. submerged fermentation**

In SSF, only a delay in  $\alpha$ -L-AFase and xylanase production was observed in high glucose concentrations containing medium (Fig. 5.2.5 and Fig. 5.2.7). In contrast, complete inhibition of enzymes production was observed in SmF medium that contain high concentrations of glucose (Fig. 2.2.1 and Fig. 5.2.3). However, the catabolic repression by glucose was overruled in the SSF most probably as a result of the gradual accumulation of the inducers due to some L-arabinose and D-xylose uptake which then triggered a further cascade in the induction of the pathway of these sugars. Furthermore, the differences in delay times for obtaining maximum  $\alpha$ -L-AFase and xylanase induction might be attributed to the different times needed by each inducer to enter and accumulate in the cell and to act on the expression of the gene and/or on the enzymatic activity (Gueimonde *et al.*, 2007). A study related to catabolite repression of the *Bacillus subtilis xyl* operon demonstrated that the glucose concentration is critical for the efficiency of glucose repression. Glucose repression appears to be most effective at low concentrations of xylose. At increased ratios of xylose over glucose, the inducer xylose is able to overcome glucose repression to a large extent (Kraus *et al.*, 1994). It was interesting to note that the  $\alpha$ -L-AFase and xylanase production was much high in SSF medium containing 150 mg g<sup>-1</sup>

glucose (15% w/w) as compared to that in SmF medium containing even lower concentration of glucose (Figs 5.2.1; 5.2.3; 5.2.5; 5.2.7).

A gradual consumption of glucose was observed in all SmF media up to the end of fermentation period (Fig. 5.2.2). In contrast, the consumption of glucose was observed to be relatively slower in the case of SSF (Fig. 5.2.6) when compared with SmF especially up to 48 h. However, rapid consumption of glucose was observed in SSF after 48 h, compare to slower and gradual glucose consumption in SmF.

Higher biomass production was observed in glucose containing SSF medium as compared to that in glucose containing SmF medium (Fig. 5.2.4 and Fig. 5.2.8). In fact, higher biomass production was also observed in glucose free controlled SSF medium as compared to SmF glucose free controlled medium. SSF was reported to produce higher microbial biomass in some studies (Lekha and Lonsane, 1994; Romero-Gomez *et al.*, 2000; Diaz-Godinez *et al.*, 2001; Aguilar *et al.*, 2001a;b; Asther *et al.*, 2002).

#### **Why SSF has the ability to minimize the catabolite repression effect on $\alpha$ -L-AFase and xylanase biosynthesis?**

These results indicated the ability of SSF system to significantly minimize the catabolic repression of  $\alpha$ -L-AFase and xylanase production by *Bacillus* sp NIOCCW19. Some authors have suggested that the reduced effect of catabolite repression in solid-state systems is due to several factors collectively, including the low water activity and absence of agitation which considerably caused slow and low processes of diffusion in solid state cultures (Ramesh and Lonsane, 1990;1991; Nandakumar *et al.*, 1999). Furthermore, the nature of solid substrate could be a very important factor to obtain a system resistant to catabolic

repression (de Souza *et al.*, 2001). This was suggested as all solid state systems described as resistant to catabolite repression were developed using wheat bran as substrate. In contrast, an SSF system with substrates such as sugar cane bagasse and corn cob has failed to resist the catabolite repression effect caused by the addition of different monosaccharides (de Souza *et al.*, 2001). However, a reasonable explanation for this phenomenon is not yet available. The close proximity of cells on surfaces such as in SSF causes physiological responses distinct from those of suspension cultures such as in SmF. Consequently, many bacterial activities such as the production of extracellular enzymes may be influenced at a genomic level (O'Reilly *et al.*, 1994; Kunst *et al.*, 1995). 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. Consequently, catabolite repression of these enzymes could also be regulated by different control mechanisms (Te Biesebeke *et al.*, 2005). This may reflect the differences in the intensity of the effect of glucose addition on  $\alpha$ -L-AFase and xylanase production by *Bacillus* sp in SSF and SmF. On the other hand, few reports indicated the ability of SSF to significantly minimize catabolic repression of  $\alpha$ -amylase production by *Bacillus licheniformis* M27 (Ramesh and Lonsane, 1991) and *Bacillus coagulans* (Babu and Satyanarayana, 1995). Furthermore, some studies also reported similar ability of SSF to minimize catabolic repression of fungal amylases (Nandakumar *et al.*, 1999), tannase (Aguilar *et al.*, 2001a;b), and pectinases (Aguilar *et al.*, 1986; Solis-Pereyra *et al.*, 1996).

Contrary to these results, however, Gessesse and Mamo (1999) observed that, the addition of glucose to wheat bran based-SSF medium resulted in mild to strong repression of xylanase production by *Bacillus* sp. Furthermore, the catabolite repression effect of glucose on xylanase production by this bacterium was found to be concentration dependent (Gessesse and Mamo, 1999). The effect of catabolite repression on enzymes production may depend also on the nature of the bacterium. Whereas, addition of 5 % glucose to wheat bran based-SSF medium of *Bacillus* sp. resulted in 26 % reduction in xylanase production (Gessesse and Mamo, 1999), addition of 0.5 % glucose concentration caused sever reduction (76 %) in xylanase production by alkaliphilic *Bacillus* sp in SmF (Gessesse, 1998). Nevertheless, few available reports indicated the ability of SSF to minimize the catabolic repression effect on xylanase production (Archana and Satyanarayana, 1997; de Souza *et al.*, 2001). However, studying the effect of carbon catabolite repression on  $\alpha$ -L-AFase production in SSF and SmF was not attempted before this study. Furthermore, studying the effect of catabolic repression on the bacterial co-production of two related enzymes such as  $\alpha$ -L-AFase and xylanase in two different growth systems (SSF and SmF) was not reported before.

### **What is the significant of the present findings?**

The xylanolytic and amylolytic enzymes are major enzymes used in production of fermentable sugars for bioethanol production (Ingledeew *et al.*, 1999; Roehr, 2001). Production of enzymes in SmF poses serious economic problem due to the effect of catabolite repression. As SSF could overcome catabolite repression effect, it could be considered for simultaneous production of xylanolytic ( $\alpha$ -L-AFase and xylanase) and amylolytic enzymes from agro-

industrial waste materials that contain easily metabolizable sugars such as glucose and fructose. Many advantages of using such fermentation system could be attained in many biotechnological applications including bioethanol production (Roehr, 2001; Sørensen *et al.*, 2006b). The advantages of using SSF may include providing low cost efficient enzymes for saccharification of biomass to fermentable sugars. Preventing feedback inhibition and catabolite repression caused by high concentration of glucose on enzymes used for saccharification and fermentation processes. Moreover, considering the use of SSF in bioethanol production during the mashing process for simultaneous production of xylanolytic and amylolytic enzymes could solve the problem of high viscosities in mashes caused by pentosans (Wang *et al.*, 1997; Ingledew *et al.*, 1999; Roehr, 2001). Furthermore, by utilizing the hemicellulosic pentose sugars a potential 30% increase in yield of ethanol can be achieved (Lawford and Rousseau, 1991; Roehr, 2001; Sørensen *et al.*, 2005; 2006b).

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# *Chapter 6*

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## SUMMARY

- ❖ Seventy three bacterial cultures capable of producing  $\alpha$ -L-AFases were isolated using enrichment technique with arabinose-containing substrates as carbon sources. In the sediment samples collected from the Mandovi estuary and Dona Paula Bay, *Bacillus* and *Pseudomonas* were the most predominant, followed by *Staphylococcus*, *Vibrio* and *Aeromonas*. *Bacillus* spp were found to be the most effective producers for  $\alpha$ -L-AFases and other xylanolytic enzymes. From the isolated bacteria, two isolates *Pseudomonas* sp NIOCCArA27 and *Bacillus* sp NIOCCW19 were selected for further studies.
  
- ❖ *Pseudomonas* sp NIOCCArA27 produced extracellular  $\alpha$ -L-AFase. The  $\alpha$ -L-AFase enzyme was isolated by ammonium sulfate (80 %) fractionation, and purified to homogeneity using size exclusion and ion exchange chromatography. The molecular mass of  $\alpha$ -L-AFase was  $\sim$  84 kDa. The enzyme had a pH optima of 7.0 and was completely stable for more than 12 h at this pH at room temperature ( $28 \pm 2^\circ\text{C}$ ). It retained almost 100% of its activity at pHs from 5.0 to 9.0 for 4 h. The activity of the enzyme decreased sharply at extreme acidic or alkaline pHs. The optimum temperature for the enzyme activity was  $65^\circ\text{C}$  at pH 7.0. The enzyme showed complete stability at  $40^\circ\text{C}$  for more than 12 h. The stability decreased when the temperatures was  $>55^\circ\text{C}$ . The enzyme retained more than 90% of its activity at  $55^\circ\text{C}$  for more then 1 h, while only 45% enzyme activity was retained after incubation at  $75^\circ\text{C}$  for 20min. For p-nitrophenyl- $\alpha$ -L-arabinofuranoside the

enzyme showed a  $K_m$  value of 0.36 and  $V_{max}$  value of 23.53  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  when the reaction was carried out at 65°C and pH 7.0. Presence of metal ion  $\text{Cu}^{2+}$  caused partial inhibition of  $\alpha$ -L-AFase activity, whereas complete inhibition was observed in the presence of  $\text{Hg}^{2+}$  and SDS. In contrast, presence of  $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  have no significant effect on the enzyme activity. The  $\alpha$ -L-AFase from *Pseudomonas* sp. exhibits high activity toward *p*NP- $\alpha$ -L-arabinofuranoside, but no activity was observed towards other *p*NP-aryl synthetic substrates. With respect to substrate specificity the  $\alpha$ -L-AFase from *Pseudomonas* sp was highly active on all  $\alpha$ -L-arabinofuranooligosaccharides. The enzyme showed a novel property in releasing arabinose from arabinan and debranched arabinan, but not from any other natural arabinose containing substrates used. This property could be of benefit if utilized for degradation of arabinans that cause haze formation in fruits juices and for production of L-arabinose - the antiglycemic sugar- from arabinans containing substrates.

- ❖ Nevertheless, *Pseudomonas* sp NIOCCara27 produces  $\alpha$ -L-AFase with desirable properties that has not been reported before. However, the enzyme was active against few substrates which included arabinan and debranched arabinan. An enzyme with broader substrate specificity which can be produce at low cost would be more desirable for industrial applications. Furthermore, *Pseudomonas* sp NIOCCara27 failed to grow under solid state fermentation (SSF) condition and produces low amounts of  $\alpha$ -L-AFase when grown on cheap agricultural residues under submerged fermentation (SmF). Therefore, another culture *Bacillus* sp NIOCCW19 was selected for further studies.

- ❖ *Bacillus* sp NIOCCW19 produced extracellular  $\alpha$ -L-AFase, when grown on a variety of agricultural residues under SSF and SmF conditions. Optimization of growth medium and conditions for the production of  $\alpha$ -L-AFase under SSF and SmF was conducted. Among the various agricultural residues studied, wheat bran was the most suitable agro-industrial residue for  $\alpha$ -L-AFase production by *Bacillus* sp NIOCCW19 in SSF as well as SmF. Several process variables were found to effect  $\alpha$ -L-AFase production by *Bacillus* sp when grown under SSF and SmF. Compare to SmF, production of  $\alpha$ -L-AFase under SSF require less nitrogen sources such as peptone and yeast extract and less inoculum size. Production of  $\alpha$ -L-AFase under SSF could be accomplished under high pH and wide range of temperature which is useful in lowering the risk of contamination. Most important, an average of 4.56 fold increase in the yield of  $\alpha$ -L-AFase was accomplished in SSF as compared to SmF. Results of this study showed that the production of  $\alpha$ -L-AFase by *Bacillus* sp NIOCC W19 are controlled in response to the physico-chemical factors, environment factors, nutritional status and culture conditions specific to the growth system.
  
- ❖ *Bacillus* sp NIOCCW19 produced extracellular  $\alpha$ -L-AFase, when grown on wheat barn as a carbon source under SSF. The  $\alpha$ -L-AFase was isolated by ammonium sulfate (55%) fractionation and purified to homogeneity using size exclusion and ion exchange chromatography. The molecular mass of  $\alpha$ -L-AFase was ~75 kDa. The enzyme had a pH optima of 8.0 and showed more than 50% relative activity at a broad range of pH ranging from pH 4 to 9. The enzyme showed almost complete stability at pHs from 6.0 to 9.0 for

more than 24 h. It also retained 84.99%, 54.39% and 76.04% of its original activity after 24 h incubation at pH 10, pH 11 and pH 5.0, respectively. The optimum temperature for enzyme activity was found at 75°C at pH 8.0. The enzyme showed complete stability at 50°C for more than 24h. It retained 92.12% and 96.8 % of its original activity at 60°C and 70°C for 14 h and 7 h, respectively. Moreover, the enzyme showed a half-life of about 7 h, 4 h and 20 min at 75°C, 80°C and 90°C, respectively. For *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside, the enzyme showed a  $K_m$  value 0.46 mM and  $V_{max}$  value of 323  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  when the reaction was carried out at 75°C and pH 8.0. The amino acid analysis of the enzyme revealed that the enzyme contained 21.6 % and 15.21 % of acidic and basic amino acids. The enzyme contains unusual high molar ratio of glycine which could be the reason of stability of the enzyme in wide range of pH. Whereas, metal ions  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , or  $\text{Co}^{2+}$ , did not enhance enzyme activity, slight increase in the enzyme activity was observed in the presence of  $\text{Fe}^{2+}$  and  $\text{Mn}^{2+}$ . However, presence of EDTA and dithiothreitol (DTT) did not affect enzyme activity. In contrast  $\beta$ -mercaptoethanol and SDS caused moderate inhibition of enzyme activity. The enzyme was active on *p*NP- $\alpha$ -L-arabinofuranoside whereas no activity was detected towards other *p*NP-aryl synthetic substrates. The  $\alpha$ -L-AFase releases arabinose by exo-acting manner, acts on all types of  $\alpha$ -L arabinofuranosyl linkages including  $\alpha$ -L-1,2 ;  $\alpha$ -L-1,3, and  $\alpha$ -L-1,5 linkages and was highly active on all  $\alpha$ -L-arabinofuranooligosaccharides. The enzyme showed broad substrate specificity and acted on arabinans, debranched arabinan, oat spelt xylan, wheat arabinoxylan and arabinogalactan and releasing high amount of

arabinose from these substrates. The biochemical properties and substrates specificity of  $\alpha$ -L-AFase *Bacillus* sp NIOCCW19 make this enzyme far more superior to all  $\alpha$ -L-AFases isolated previously from other *Bacillus* spp and many fungi and bacteria. The characteristics of this enzyme make this enzyme potentially very effective for industrial applications.

- ❖ The effect of  $\alpha$ -L-AFase and xylanase produced by *Bacillus* sp NIOCCW19 on some hemicelluloses was investigated. Treatment of wheat arabinoxylan and oat spelt xylan with  $\alpha$ -L-AFase resulted in the release of reducing sugars of 339.6  $\mu\text{g/ml}$  and 61.2  $\mu\text{g/ml}$ , respectively. Treatment of wheat arabinoxylan and oat spelt xylan with xylanase resulted in the release of reducing sugars of 272.4  $\mu\text{g/ml}$  and 268.2  $\mu\text{g/ml}$ , respectively. When both enzymes  $\alpha$ -L-AFase and xylanase applied together, 928  $\mu\text{g/ml}$  and 484.5  $\mu\text{g/ml}$  reducing sugars were released from wheat arabinoxylan and oat spelt xylan, respectively. It was observed that presence of  $\alpha$ -L-AFase in combination with xylanase resulted in hydrolysis enhancement of about 51.63 % and 47.09 % in releasing reducing sugars from wheat arabinoxylan and oat spelt xylan, respectively. The increased rates of hydrolysis of these substrates was apparently due to catalytic synergy, as apposed to enhanced activity of xylanase conferred by the presence of  $\alpha$ -L-AFase. The substrates hydrolysis enhancements caused by the action of the  $\alpha$ -L-AFase produced by *Bacillus* sp NIOCCW19 was higher than that obtained from  $\alpha$ -L-AFases produced by other bacteria.
- ❖ An attempt has been made to develop suitable chemo-enzymatic treatment for production of fermentable sugars from sugar cane bagasse. The chemical

pretreatment of raw bagasse resulted in sugar yields of 16.46%, 5.73% and 8.82% of total sugar initially present in raw bagasse that was pretreated with diluted HCl; H<sub>2</sub>O<sub>2</sub> and NaOH, respectively. The enzymatic treatment of acid pretreated bagasse resulted in sugar yield of 54.358% of total sugar initially present in bagasse. This sugar yield was obtained by the action of cellulases enzymes only. The overall sugar yield of acid pretreatment and enzymatic pretreatment in this case was 72% of the total sugar initially present in bagasse. Treatment of hydrogen peroxide pretreated bagasse with  $\alpha$ -L-AFase, xylanase and cellulases, resulted in sugar yields of 1.18%, 8.57% and 20.13%, respectively, of total sugars initially present in bagasse. An overall sugar yield of 42.8 % of the total sugar initially present in bagasse was obtained by hydrogen peroxide pretreatment and enzymatic treatment with mixture of  $\alpha$ -L-AFase, xylanase and cellulases. Treatment of NaOH pretreated bagasse with  $\alpha$ -L-AFase, xylanase and cellulases, resulted in sugar yields of 1.48%, 12.45%, and 43.35% respectively, of total sugars initially present in bagasse. An overall sugar yield of 81.2 % of the total sugar initially present in bagasse was obtained by NaOH pretreatment and enzymatic treatment with mixture of  $\alpha$ -L-AFase, xylanase and cellulases. Comparing, the overall sugar yields of chemical pretreatments and enzymatic treatments revealed the superiority of the use of a combination of NaOH pretreatment and enzymatic treatment with  $\alpha$ -L-AFase, xylanase and cellulases in producing the highest amount of fermentable sugars from sugar cane bagasse. Furthermore, the saccharification yield obtained was higher than that obtained in previous studies from sugar cane bagasse. The utilization of such chemical and enzymatic treatment could have economic

and environmental benefits. The synergistic effect of  $\alpha$ -L-AFase and xylanase was further confirmed by their action on hemicelluloses released from NaOH and H<sub>2</sub>O<sub>2</sub> pretreated bagasse. Taken together, these results reinforced that the cooperative action of  $\alpha$ -L-AFase and xylanase is essential for complete hydrolysis of arabinoxylans present in hemicelluloses, especially those containing large amount of arabinose side chains.

- ❖ The effect of catabolite repression on the biosynthesis of  $\alpha$ -L-AFase and xylanase by *Bacillus* sp NIOCCW19 under SSF and SmF was investigated.  $\alpha$ -L-AFase production was reduced by 17.4% and 24.92% in SmF medium with low glucose concentrations of 5 and 10 mg ml<sup>-1</sup>, respectively. The  $\alpha$ -L-AFase production was reduced by more than 90% in SmF medium containing glucose at concentrations of 50 and 150 mg ml<sup>-1</sup>. In contrast, slightly slower rate of  $\alpha$ -L-AFase synthesis up to 48 h was observed in SSF medium supplemented with low glucose concentrations as compared to that in glucose free controlled SSF medium. By the end of fermentation period, however,  $\alpha$ -L-AFase production was slightly higher in these SSF media. The presence of high glucose concentration resulted in a delay in biosynthesis of  $\alpha$ -L-AFase in SSF. However, the  $\alpha$ -L-AFase production in SSF medium with high glucose concentration was increased gradually after 48h. Interestingly, compare to glucose free SSF medium at 120 h,  $\alpha$ -L-AFase production was increased by 19.86% and 28.93% in SSF medium supplemented initially with glucose at 50 mg g<sup>-1</sup> and 150 mg g<sup>-1</sup>, respectively. Xylanase production was not reduced in the presence of

glucose at low concentrations ( $5 \text{ mg ml}^{-1}$  and  $10 \text{ mg ml}^{-1}$ ) in SmF media. Interestingly, presence of glucose at concentration of  $5 \text{ mg ml}^{-1}$  in SmF media enhanced xylanase production by 10.65%. However, severe reduction in xylanase biosynthesis was observed in SmF medium with higher concentrations of glucose. The presence of low glucose concentrations in SSF medium did not reduce xylanase production or delay the synthesis of the enzyme. Furthermore, compare to glucose free SSF medium at 120 h, xylanase production was increased by 9.51% and 21.15% in SSF medium supplemented initially with glucose at  $5 \text{ mg g}^{-1}$  and  $10 \text{ mg g}^{-1}$ , respectively. The presence of glucose in high concentration ( $50$  and  $150 \text{ mg g}^{-1}$ ) resulted in a delay in xylanase biosynthesis. However, a gradual increase in xylanase production was observed after 48h of fermentation period in these media. Compare to glucose free SSF medium at 120h, xylanase production was increased by 28.75% and 33.57% in SSF medium supplemented initially with glucose at  $50 \text{ mg g}^{-1}$  and  $150 \text{ mg g}^{-1}$ , respectively. In SmF, the biosynthesis of  $\alpha$ -L-AFase by *Bacillus* sp seems to be more effected by the presence of low concentration of glucose when compared to xylanase. However, at high glucose concentration production of both the enzymes ( $\alpha$ -L-AFase and xylanase) were severely inhibited in SmF. In contrast, only a delay in  $\alpha$ -L-AFase and xylanase production was observed in SSF medium containing high concentrations of glucose. However, it was interesting to note that the production of  $\alpha$ -L-AFase and xylanase increased significantly with the increase in glucose concentration and enzymes production reached levels that were even higher than that in glucose free controlled medium. Hence, the catabolic repression effect of glucose on

$\alpha$ -L-AFase and xylanase production was overruled in SSF. The results indicated the ability of SSF system to significantly minimize the effect of catabolic repression on  $\alpha$ -L-AFase and xylanase biosynthesis by *Bacillus* sp NIOCCW19. As SSF could overcome catabolite repression effect, it could be considered for simultaneous production of xylyanolytic ( $\alpha$ -L-AFase and xylanase) and amylyolytic enzymes for use in many industrial applications such as bioethanol production. As far we are concerned, this study is the first contribution towards understanding the effect of catabolite repression on  $\alpha$ -L-AFase biosynthesis by bacterium grown under SSF and SmF.

### **Areas of further research**

Achieving efficient breakdown of the plant cell wall polysaccharide hemicelluloses and pectins represents an important and lucrative goal for biotechnologists. For that, further research has to be carried out to explore many aspects of  $\alpha$ -L arabinofuranosidases, in much detail. Further, studies on the synergistic effects of the robust enzyme on the action of other hemicellulases and pectinases that already exist as commercial enzymes may lead to improvement of many existing industrial products. Understanding how these enzymes interact and act on lignocelluloses and the relationship between their structure and function at molecular level are other aspects that need to be studied. Moreover, isolation and characterization of such robust  $\alpha$ -L-AFases and genes encoded for these enzymes 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. Manipulation and genetics engineering of bacteria for obtaining complete and a more efficient lignocellulose degrading enzymes system including  $\alpha$ -L-AFases genes will be a novel path into complete saccharification system, which is required for many technologies including ethanol production. Furthermore, chemical treatments in some industries such as paper and pulp bleaching and ethanol production which cause environmental problems could be reduced or replaced by using an efficient hemicellulose-degrading enzymes system.

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## **List of Publications**

- ❖ **Numan MTH** and **Bhosle NB** (2006).  $\alpha$ -L-Arabinofuranosidases: the potential applications in biotechnology. *J Ind Microbiol Biotechnol* 33: 247-260.
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- ❖ **Khandeparker R**, **Numan MTH**, **Mukherjee B**, **Satwekar A** and **Bhosle NB** (2008). Purification and characterization of  $\alpha$ -L-arabinofuranosidase from *Arthrobacter* sp MTCC 5214 in solid-state fermentation. *Process Biochem* 43:707-712.

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## $\alpha$ -L-Arabinofuranosidases: the potential applications in biotechnology

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**Abstract** Recently,  $\alpha$ -L-arabinofuranosidases (EC3.2.1.55) have received increased attention primarily due to their role in the degradation of lignocelluloses as well as their positive effect on the activity of other enzymes acting on lignocelluloses. As a result, these enzymes are used in many biotechnological applications including wine industry, clarification of fruit juices, digestion enhancement of animal feedstuffs and as a natural improver for bread. Moreover, these enzymes could be used to improve existing technologies and to develop new technologies. The production, mechanisms of action, classification, synergistic role, biochemical properties, substrate specificities, molecular biology and biotechnological applications of these enzymes have been reviewed in this article.

**Keywords**  $\alpha$ -L-arabinofuranosidases · Lignocelluloses · Synergistic role · Classification · Applications

### Introduction

Lignocelluloses of plant cell walls are composed of cellulose, hemicellulose, pectin and lignin. Hemicelluloses 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. Many enzymes are involved in the degradation of these polymeric substrates [129]. L-arabinofuranosyl residues are widely distributed in these polymers as side chains. The presence of these side chains restricts the enzymatic hydrolysis of hemicelluloses and pectins [93, 99, 101]. Further, it also represents a formidable technological barrier that retards the development of various industrial processes [99]. 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 [72, 110].

The  $\alpha$ -L-arabinofuranosidases ( $\alpha$ -L-AFases) are accessory enzymes that cleave  $\alpha$ -L-arabinofuranosid linkages and act synergistically with other hemicellulase and pectic enzymes for the complete hydrolysis of hemicelluloses and pectins [77, 113]. These enzymes warrant substantial research efforts because they represent potential rate-limiting enzymes in the degradation of lignocelluloses from agricultural residues [99]. The application of  $\alpha$ -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 [44, 46, 74], synthesis of oligosaccharides [94, 95] and pretreatment of lignocelluloses for bioethanol production [100, 101]. Considering the potential and future prospects of  $\alpha$ -L-AFases, this paper reviews the various aspects of these enzymes with emphasis on their potential for biotechnology.

### Hemicelluloses and pectins

Hemicelluloses and pectins are the matrix polysaccharides of the plant cell wall. They account for 25–35% of lignocellulose biomass [99]. The hemicellulose xylans contain  $\beta$ -1,4-linked D-xylose backbone [30]. In many plants, the xylan backbone is substituted by different side chains with L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl and glucuronic residues [1, 30]. 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 [99, 100]. Similarly, arabinoxylans are found in the cell walls of the cereal plants and grasses belonging to the family Gramineae [1, 70]. They contain xylan backbone that is partially substituted at intervals with  $\alpha$ -L-arabinofuranose residues [1]. Moreover, wheat arabinoxylan also contains other substituents as shown in Fig. 1 [1,

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## functional xylanases and their potential use in biotechnology

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**Abstract** Plant cell walls are comprised of cellulose, hemicellulose and other polymers that are intertwined. This complex structure acts as a barrier to degradation by single enzyme. Thus, a cocktail consisting of bi and multifunctional xylanases and xylan debranching enzymes is most preferred combination for the efficient utilization of these complex materials. Xylanases have prospective applications in the food, animal feed, and paper and pulp industries. Furthermore, in order to enhance feed nutrient digestibility and to improve wheat flour quality xylanase along with other glycohydrolases are often used. For these applications, a bifunctional enzyme is undoubtedly much more valuable as compared to monofunctional enzyme. The natural diversity of enzymes provides some candidates with desired bifunctional activity. Nevertheless most resulted from the in vitro fusion of individual enzymes. Here we present bifunctional xylanases, their evolution, occurrence, molecular biology and potential uses in biotechnology.

**Keywords** Xylanase · Bifunctional · Enzyme · Biotechnology · Hemicellulose · Cellulase

### Introduction

Cellulose, hemicellulose, and lignin are the major components of plant cell walls [89]. When combined, they form a support framework for plants and defend against the invasion of aggressors. To successfully invade or live on

plant tissues, microorganisms have to synthesize a number of different enzymes in order to hydrolyze cellulose or hemicellulose. Thus, to effectively degrade the plant cell wall complex, many microorganisms develop a cell associated multiprotein complex, called cellulosome [43] or xylosome [47], which contains cellulases, xylanases, and cellulose-binding factors. Another strategy is to induce the multifunctionalization of certain enzymes to hydrolyze different kinds of substrates. In addition to its importance in allowing microorganisms to invade plants and degrade plant residues, xylanase also has prospective applications for the food, animal feed, and paper and pulp industries. Furthermore, for the enhancement of feed nutrient digestibility [54, 56, 68, 93] and improvement of wheat flour quality [30] xylanases and  $\beta$ -(1,3-1,4)-glucanases are used at the same time. Similarly, bioethanol production requires efficient saccharification for degrading plant cell walls efficiently to fermentable sugars. Therefore, by biologically converting plant cell walls to fermentable sugars for fuel (e.g., ethanol), we could obtain not only economic but also environmental benefits, such as the reduction of greenhouse gas emission. A critical factor concerning the cost of this process is the presence of efficient, cheap cellulases and xylanases to achieve this goal in a single step.

Utilization of pentoses sugars present as hemicellulosic fraction of lignocellulosic biomass is a major step towards reducing production cost of bioethanol. Moreover, presence of xylanases along with cellulases in the fermentation mixture during mashing process can solve the problem of viscosity caused by pentosans [75]. For these applications, a bifunctional enzyme is undoubtedly more valuable than a single enzyme. In this review we will discuss the recent development in bifunctional xylanases with respect to their occurrence in microorganisms, molecular biology, genetic and protein engineering, and their importance for industry.

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## Purification and characterization of $\alpha$ -L-arabinofuranosidase from *Arthrobacter* sp. MTCC 5214 in solid-state fermentation

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### ABSTRACT

*Arthrobacter* sp. MTCC 5214 produced an  $\alpha$ -L-arabinofuranosidase when grown on solid-state fermentation (SSF). The enzyme was purified 19-fold using ion exchange and gel filtration chromatography. The enzyme had an apparent molecular mass of ~97 kDa. With *p*-nitrophenol arabinofuranoside as the substrate, the enzyme exhibited a  $K_m$  of 0.3 mM, and a  $V_{max}$  of 3.34  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup>. The enzyme had optimum activity at pH 8 and 80 °C. At pH 8.0 the enzyme was stable at 50 and 60 °C for 24 h, whereas it retained 90% of its activity after incubation at 70 °C. Metal ions such as Co<sup>2+</sup> and Fe<sup>2+</sup> induced, whereas Hg<sup>2+</sup> inhibited the activity of the enzyme. To our knowledge, this is the first report on the production of  $\alpha$ -L-arabinofuranosidase by a bacterium grown on solid-state fermentation.

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422

### 1. Introduction

Hemicelluloses, the second most common polysaccharides in nature, represent about 20–35% of lignocellulosic biomass [1]. L-Arabinosyl residues are widely distributed in hemicelluloses. They constitute monomeric or oligomeric side chains on the  $\beta$ -(1,4)-linked xylose or galactose backbones in arabinoxylans and arabinogalactans, and are the core in arabinans forming  $\alpha$ -(1,5)-linkages [2,3]. These side chains restrict the enzymatic hydrolysis of hemicelluloses [4]. The  $\alpha$ -L-arabinofuranosidases ( $\alpha$ -L-arabinofuranoside arabinofuranohydrolase, EC: 3.2.1.55,  $\alpha$ -L-AFase) are exo-type enzymes, which hydrolyze terminal non-reducing  $\alpha$ -L-arabinofuranosyl groups from L-arabinose containing polysaccharides. These enzymes can hydrolyze (1,2), (1,3) and (1,5)- $\alpha$ -L-arabinofuranosyl linkages of arabinans. The  $\alpha$ -L-AFases are part of the microbial xylanolytic systems necessary for the complete breakdown of heteroxylans [4–7].

In recent years,  $\alpha$ -L-AFases have received considerable attention because of their potential applications in various agro-industrial processes including efficient conversion of hemicellulosic biomass to fuels and chemicals, delignification of pulp, efficient use of plant

materials for animal feed, and hydrolysis of grape monoglycosides during wine fermentation [8–13]. In view of this, there is a need to develop suitable  $\alpha$ -L-AFases for use in the conversion of hemicelluloses to fermentable sugars which could be used for subsequent production of fuel ethanol, and other valuable chemicals [12,13]. Although  $\alpha$ -L-AFases are produced by bacteria and fungi, only a few have been purified and characterized [14–20].

Bacterial  $\alpha$ -L-AFases are mostly isolated from the culture under submerged cultivations. Nevertheless, a few fungal strains are known to produce  $\alpha$ -L-AFases when grown under solid-state fermentation (SSF) [17,21,22]. Recently, SSF has gained interest because it offers many economical and environmental advantages for the production of many enzymes [23]. For SSF needs simpler equipments and plants with low requirements. This results in lower operation cost and high productivity [24,25]. Therefore there is a need of bacteria that can be cultivated under SSF for the production of enzyme. In view of this, we scanned several marine bacterial strains for production of  $\alpha$ -L-AFase when grown under SSF using wheat bran as a sole source of carbon and energy. One of the culture *Arthrobacter* sp. MTCC 5214 produced an  $\alpha$ -L-AFase in substantial amount when grown under SSF. In this paper, we present data on the purification, purification and characterization of  $\alpha$ -L-AFase from *Arthrobacter* sp. grown under SSF.

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