Alkaline and Thermostable Xylanases of Potential Industrial Importance from Alkaliphiles

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ABSTRACT

Three xylanase producing alkaliphilic bacterial strains designated AR-009, AR-135 and AR-22-1, were isolated from Lake Arenguadie, an alkaline soda lake in Ethiopia. AR-009 and AR-135 were identified as strains of the genus *Bacillus* and *Micrococcus*, respectively, while AR-22-1 remains unidentified. The enzyme from all the 3 strains were purified following standard protein purification procedures, and the molecular weight of each enzyme was estimated using SDS-PAGE.

Two xylanases designated as xylA and xylB, having molecular weights of 23 and 48 kD, respectively, were purified from the cell free culture supernatant of *Bacillus* sp. AR-009. XylA was optimally active at pH 9 while xylB showed optimum activity in the pH range of 9 to 10 and stable from pH 5 to 11. The optimum temperature for the activity of xylA was 70°C at pH 8 and 60°C at pH 9. On the other hand xylB was optimally active at 70°C at pH 8 and 75°C at pH 9. Both enzymes showed good stability at 60°C and at a pH of 8 and 9.

AR-135 xylanase was optimally active at 55°C and a pH of 7.5 - 9.0. Over 60% of the maximum activity was displayed at pH 11. Its thermal stability above 40°C was very low. The optimum temperature and pH for the activity of AR-22-1 xylanase was 70°C and 8.0 to 9.5, respectively. The enzyme was stable in a broad pH range and showed good stability up to 60°C at pH 8 and 9

Agar immobilized cells of *Bacillus* sp. AR-009 were used for xylanase production with a view of finding cheap ways of enzyme production. In a batch culture maximum enzyme production was observed after 48 h and remained high up to 72 h. In repeated batch cultivation immobilized cells produced appreciable level of xylanase activity in 7 consecutive batches with out any significant decline in productivity. For continuous xylanase production immobilized cells were packed in a jacketed glass column and sterile medium was continuously pumped. A stable continuous production of xylanase was observed over a period of one month. The volumetric productivity of the continuous culture was 17 fold higher than the batch culture using free cells.

Another method of enzyme production investigated was the use of solid state fermentation. *Bacillus* sp. AR-009 produced high level of xylanase activity when grown using solid state fermentation (SSF) with wheat bran serving as a substrate. Xylanase production was highest at a wheat bran to moisture ratio of between 1:0.5 to 1:1.5 and a Na_2CO_3 concentration of 10% (w/w). No significant effect was observed on xylanase production when wheat bran is supplemented with peptone, tryptone, and yeast extract, thus avoiding the need to supplement wheat bran with expensive media. The ability of the organism to produce high titre xylanase activity at alkaline pH and lower wheat bran to moisture ratio could have a potential advantage in minimising the risk of contamination. In addition, because the enzyme can be extracted using minimum volume of liquid, the cost of down stream processing during product upgrading and the cost of waste treatment steps can be greatly reduced. The use of SSF for the production of xylanase by *Bacillus* sp. AR-009 could therefore lead to substantial reduction in the over all cost of enzyme production.

I. INTRODUCTION

1. Overview

Next to cellulose xylan is the most abundant biological polymer on earth. Xylan can be hydrolysed to its component sugars using mineral acids or enzymes. However, from the application point of view, enzymatic hydrolysis offers several advantages. A diverse group of microorganisms produce xylan degrading enzymes (xylanases). In recent years such enzymes found important application for the bleaching of kraft pulp, as animal feed supplement, for the hydrolysis of lignocellulosic biomass, and in many other industrial processes. For many of these applications xylanases that are optimally active at alkaline pH are required. On the other hand, most of xylanases known to date are optimally active at or below neutral pH, showing the need for new xylanases which are optimally active in the alkaline range.

To obtain enzymes suited for a given application condition, at present two alternatives can be followed: 1) improving existing enzymes through protein engineering; or 2) screening for novel enzymes from new microbial strains. With our current knowledge of protein structure to function relationship it is very difficult to design an enzyme that would fulfil the requirement(s) for a particular application condition through protein engineering alone. Therefore, isolation of new microbial strains from nature is considered to be the best option.

In the search for novel xylanases in the last two decades attention has been mainly focused on the isolation of xylanolytic microorganisms from extreme environments. One such source is naturally occurring alkaline habitats which offer a good opportunity for the isolation of novel alkaliphilic microbial strains that could produce alkaline active xylanases suitable for different applications.

2. Alkaliphilic microorganisms

Organisms growing at pH values greater than 8, with an optimum between pH 9-10, are called alkaliphiles. Those alkaliphiles capable of growing at neutral pH, with an optimum in the alkaline range, are called facultative alkaliphiles while those that do not grow at neutral pH are called obligate alkaliphiles. Understanding the mechanism for

growth at alkaline pH, a condition lethal to most other organisms, and the potential industrial applications of enzymes derived from such organisms are the main reasons for the increased attention given to the study of alkaliphilic microorganisms.

2.1. Ecology and diversity of alkaliphiles

Alkaliphilic microorganisms have been isolated from natural and man-made alkaline habitats, neutral soil, and in some cases even from acidic soil (Horikoshi and Akiba, 1982; Grant *et al.*, 1990; Jones *et al.*, 1994). The great majority of alkaliphilic species isolated from neutral soil so far are members of the genus *Bacillus* which indicate the limited diversity of alkaliphiles in such habitats (Grant *et al.*, 1990; Jones *et al.*, 1994). However, occasionally alkaliphilic strains belonging to the genera *Micrococcus, Pseudomonas, Paracoccus, Aeromonas, Corynebacterium,* and *Streptomyces* have been isolated from neutral soil at different parts of the world (Horikoshi and Akiba, 1982; Jones *et al.*, 1994). The occurrence of alkaliphiles in neutral and acidic soil is expected to be a result of localized transient alkaline conditions created due to biological activities, such as ammonification, sulphate reduction, and photosynthesis (Langworthy, 1978).

Another source of alkaliphiles are man-made alkaline habitats. Some industrial activities, such as cement manufacture, pulp and board production, food processing, electroplating etc. result in the release of alkaline effluents which, up on continuous release in to the surrounding environment, create a stable alkaline habitat. Over time this could create a favourable condition for the growth and dominance of alkaliphilic species. Though very little microbiological work has been done in such habitats, organisms belonging to the genera *Exiguobacterium* (Gee *et al.*, 1980; Collins *et al.*, 1983) and *Bacillus* (Yang *et al.*, 1995) have been isolated from such man-made alkaline habitats. Some of these organisms were found to produce alkaline active enzymes of potential application (Yang *et al.*, 1995).

A large diversity of alkaliphiles are expected to occur in naturally occurring alkaline habitats. Such environments are found scattered in different parts of the world, though they are few in number and are not evenly distributed on the surface of the earth

(Grant, 1992). The different natural alkaline habitats are known to differ in their chemical composition and their biological diversity. On the basis of their chemical composition these habitats are divided into two broad categories as high calcium and low calcium alkaline habitats (Grant, 1992).

2.1.1. High calcium alkaline habitats

The high Ca^{2+} habitats are ground water springs which are usually diluted and very alkaline, having pH in excess of 11. They are characterized by high concentration of Ca^{2+} in the form of $Ca(OH)_2$. These ground water springs are also characterized by low concentration of carbon, nitrogen, phosphate, and a high concentration of Fe^{2+} thus creating a reducing condition (Grant, 1992: Jones *et al.,* 1994).

Some microbiological studies conducted on alkaline ground water springs of Oman showed a low microbiological load with a viable count of 10^1 to 10^4 cfu/ml organotrophic bacteria (Jones *et al.,* 1994). All the strains isolated were identified as members of the genera commonly found in the surrounding soil and water habitats, thus leading to the speculation that they may be contaminants from the surrounding environment rather than being indigenous population of these springs (Jones *et al.,* 1994). Generally very limited microbiological studies have so far been conducted in these habitats.

2.1.2. Low calcium alkaline habits

Another class of naturally occurring alkaline habits are the alkaline soda lakes. These habitats are characterized by high concentration of carbonate and bicarbonate, and a stable alkaline pH, usually between 10-11. A unique combination of climatic, topological, geological, and geographical conditions are believed to contribute to the formation of these habitats (Grant 1992; Jones *et al.,* 1994). Typical examples of such habitats are the alkaline soda lakes of the Great Rift Valley in East Africa. These lakes are characterized by having a closed drainage basin, high marginal relief for refill of water by run off, and high rate of evaporation exceeding the rate of water refill which lead to the high concentration of ions. The surrounding catchement area is characterized by having very low Ca^{2+} and Mg^{2+}. Carbonate/ bicarbonate

concentration is in great molar excess of the Mg^{2+} and Ca^{2+} ions leading to the precipitation of any Mg^{2+} and Ca^{2+} ions initially present to insoluble carbonate salts.

The East African alkaline soda lakes are interesting in that they are highly productive, with the highest primary productivity ever recorded for a natural habitat (Talling *et al.*, 1973). The very high primary productivity observed is a result of the unlimited availability of CO_2 (in the form of HCO^-_3/CO^-_3 reserve) and high light intensity in the region (Grant *et al.*, 1990). In some of these lakes the main photosynthetic organisms belong to the genus *Spirulina (Arthrospira)*, a cyanobacteria very well known for its high protein content and other good nutritional qualities as human food and animal feed (Belay *et al.*, 1993; 1996). Other cynobacteria belonging to the genera *Cyanospira (Anabaenopsis)* and *Chlococcus* are also known to have significant photosynthetic contribution (Florenzano *et al.*, 1985; Jones *et al.*, 1994). In addition, in some of the soda lakes the anoxygenic photosynthetic bacteria *Ectothiorhodospira* is abundant and expected to have important photosynthetic contribution, at least in some conditions (Jones *et al.*, 1994). In diluted alkaline soda lakes eukaryotic algae, such as the diatoms belonging to the genera *Nitzschia* and *Navicula,* are also important contributors of primary productivity (Hecky and Kilham, 1973).

The very high photosynthetic productivity and hence the great abundance of organic matter in alkaline soda lakes leads to the assumption that a large variety of heterotrophic microorganisms (aerobic and anaerobic) to be present. Such organisms play a crucial role in nutrient recycling. A study in the heterotrophic microbial population of some Kenyan soda lakes for a period of one year showed the presence of a stable population of 10^6-10^7 bacterial cells/ml with total count using epiflorecent microscopy and 10^4-10^5 cfu/ml using viable count (Grant *et al.*, 1990). Sequencing of the 16S RNA gene from some selected strains of heterotrophic alkaliphiles from some selected Kenyan alkaline soda lakes showed the presence of a wide diversity of phylogenetic groups representing many taxa (Duckworth *et al.*, 1996). However, to date extremely little microbiological work has been done on the study of heterotrophic bacterial populations from these alkaline soda lakes.

From the limited study conducted so far species belonging to archaea and bacteria have been described. The most common archaea isolated from these habitats belong to the genera *Natronobacterium* and *Natronococcus* (Grant 1989a; 1989b). Species belonging to the genera *Bacillus* and *Bogoriella* (Groth *et al.*, 1997), *Spirochaeta* (Zhilina *et al.*, 1996) have also been described. On top of the limited effort made up to now to isolate alkaliphiles from alkaline soda lakes, the situation may be further complicated by failure of many species of microorganisms to grow using commonly used microbiological media. Currently some experts estimate that only a small fraction of the microbial population expected to exist on earth is cultivable using commercially available media (Hugenholtz and Pace, 1996; Pace, 1997).

2.2. Physiology of alkaliphiles

Life at alkaline pH imposes a number of physiological problems. Understanding the mechanisms of survival at alkaline pH has been an interesting area of study. How do alkaliphiles meet their bioenergetic demand at very alkaline growth pH? How do they respond to fluctuation in pH and how do they maintain their intracellular pH lower than the external milieu? What biochemical attributes do alkaliphiles have which their neutralophilc counterparts lack, that enable them grow at alkaline pH? How do some alkaliphiles (the facultative alkaliphiles) grow at neutral as well as alkaline pH while others (obligate alkaliphiles) fail to grow at neutral pH? Over the years many researchers have been addressing these and other questions. In almost all cases alkaliphilc *Bacillus* strains have been used as model organisms. Though a lot more still remain to be known, from such studies many significant and interesting understanding of the physiology of alkaliphilic growth have emerged, some of which is discussed below. Given the wide spread occurrence of *Bacillus* in all habitats on the surface of the earth, it is not yet clear whether the alkaliphilic *Bacillus* species known so far (almost all isolated from neutral soil) share homologous biochemical apparatus with other strictly alkaliphilic genera isolated from naturally occurring alkaline habitats.

2.2.1. Energy generation

Alkaliphiles are known to maintain their cytoplasmic pH about 2 or more units lower than the external pH. From the bioenergetic point of view this sharply contrasts with

the standard chemiosmotic theory where the external pH is maintained lower (acidic) due to proton pumping by the respiratory chain of the membrane. Because of this difference, one hypothesis was forwarded stating that ATP synthesis in alkaliphiles could be coupled to Na^+ pumping instead of protons (Hoffman *et al.*, 1990). This was in part based on the observation that Na^+ is absolutely required for growth of alkaliphiles. Secondly a Na^+ driven ATP synthase has been earlier described from marine microorganisms (Laubinger and Dimroth, 1988), thus leading to the speculation for a similar mechanism in alkaliphiles. However, up to now no Na^+ driven ATP synthase has been isolated from any alkaliphile (Krulwich, 1995).

The F_1F_o-ATP synthase of alkaliphilic *Bacillus* strains was isolated in different laboratories and characterized (Hoffman and Dimroth, 1990; Hicks and Krulwich, 1990). Immunological and other biochemical characterization of the protein showed no major difference from their neutralophilic counterparts. Moreover, ATP synthesis by liposome bound alkaliphile F_1F_o served as proton pumps with out any sign of Na^+ pumping (Hicks and Krulwich, 1990: Hoffman and Dimroth, 1991; Hicks and Krulwich, 1995). This showed that ATP synthesis in alkaliphiles is proton driven. However, the exact mechanism of proton driven ATP synthesis by alkaliphilic ATP synthase remains unknown (Krulwich, 1995). A number of hypothesis have been forwarded so far, though none of which experimentally proved yet.

2.2.2. pH homeostasis

Another challenge alkaliphiles face is the problem of pH homeostasis. Both alkaliphiles and neutralophiles have the capacity to maintain their cytoplasmic pH lower when challenged with increasing pH at the external milieu (Booth, 1985; Krulwich *et al.*, 1997). However, at pH values of around 8.5 neutralophiles fail to survive while obligate and facultative alkaliphiles show optimum growth between pH 9 -10. At this alkaline pH, alkaliphiles maintain their cytoplasmic pH about two or more units lower than the external pH (Krulwich, 1995; Krulwich *et al.*, 1997). What mechanism is involved in maintaining the intracellular pH about 2 units or more lower than the external pH? Both passive and active mechanisms for the control of cytoplasmic pH have been suggested.

2.2.2.1. Passive mechanisms based on cell wall chemistry

Since the cell wall is in direct contact with the external alkaline medium, some workers proposed that the cell wall could some how play a role in maintaining the observed pH gradient (Aono and Horikoshi, 1993; Aono *et al.,* 1995). In agreement to this hypothesis, the cell wall of the facultative alkaliphilic *Bacillus* sp. strain C-125 was found to contain a high amount of acidic polymers, teichuronic acid and a glutamate rich teichuropeptide (Aono and Horikoshi, 1993; Aono *et al.,* 1995). The presence of these negatively charged polymers on the cell wall led to the assumption that the influx of hydroxyl ions be prevented to some extent by a repulsive force of the negatively charged cell wall. Another possibility is that the cell wall could bind to positively charged cations thus creating a repulsive force for the out flux of protons from the cytoplasm (Krulwich *et al.,* 1997). In support of this hypothesis, it was shown that mutants derived from the facultative alkaliphile *Bacillus sp C-125* lacking these polymers failed to grow at alkaline pH (Aono and Ohtani, 1990). On the other hand, analysis of the cell wall composition of other alkaliphilic *Bacillus* strains in other laboratories showed no major difference in the amount of charged polymers on the cell wall from neutralophilic bacilli (Guffanti and Krulwich, 1994), casting some doubts at least on the universality of the contribution of the cell wall in maintaining the observed pH gradient. Even if the cell wall and cell membrane of alkaliphiles may passively contribute to pH homeostasis in alkaliphiles, the observed pH gradient with increasing alkaline external milieu can not be solely explained by passive mechanisms alone, suggesting for the involvement of active mechanisms.

2.2.2.2. Active mechanisms

At present there is substantial evidence showing that the proton gradient generated by the respiratory chain of alkaliphiles, in addition to driving the synthesis of ATP via the F_1F_o-complex, is also involved in maintaining low cytoplasmic pH, drive flagellar motility and ion and solute transports (Krulwich, 1995; Sugiyama, 1995; Krulwich *et al.,* 1997). Central to this are the Na^+-H^+ antiports and the different symports.

At least three different types of Na^+-H^+ antiports have been identified from alkaliphilic *Bacillus* strains (Kitada *et al.*, 1994). These antiports transfer protons inward for an exchange of sodium from the inside thus increasing the concentration of protons in the intracellular side. Similar antiports have also been found as important components for pH homeostasis in neutralophilic strains when they are subjected to higher growth pH (Cheng *et al.*, 1994). However, while the antiports from neutralophiles can use Na^+ or K^+ ions, those from alkaliphiles are totally dependent on Na^+ (Krulwich *et al.*, 1997), which may also explain the absolute requirement for Na^+ for the growth of alkaliphilies.

Genes coding for H^+/Na^+ antiport proteins have been cloned from alkaliphilic *Bacillus* strains (Ito *et al.*, 1997) and the role of the proteins these genes code in ion transport was demonstrated in vitro (Krulwich *et al.*, 1997). Additionally mutants unable to synthesize a functional aniporter protein were found to lose their ability to grow at alkaline pH (Kitada *et al.*, 1989; Hamamoto *et al.*, 1994) indicating the central role these proteins play in the survival of alkaliphiles. However, the efficiency by which alkaliphiles maintain their cytoplasmic pH may not be explained by the action of antiport proteins known so far alone.

In order to maintain the system the Na^+ accumulating at the outside of the membrane due to the action of the antiport proteins must enter back to the cell interior. This inward movement of Na^+ is accomplished by the help of different solute symports, membrane proteins that couple the co-transport of solutes and Na^+ toward the interior (Krulwich and Guffanti, 1992). Amino acids, sugars, and other molecules are known to be transported in this way. Additional entry routes for Na^+ through specific pH induced channels have also been proposed, especially at high growth pH (Krulwich, 1995; Krulwich *et al.*, 1997). However, no such structures have so far been isolated.

3. Hemicelluloses

The term hemicellulose represents a heterogeneous group of plant cell wall polysaccharides which are alkali soluble, linear or branched polymers, precipitable by alcohols from aqueous solutions, easily hydrolyzed by mineral acids, and give a variety

of sugars upon hydrolysis (Wikie, 1983). Xylan, having a β-1,4 -linked xylopyranoside residues as its backbone, is the major component of hemicelluloses. Unlike cellulose, which is a homopolymer made of identical repetitive units of a β- -1,4-linked D-glucose residues, xylan is a heteropolymer usually having a branched chain. Branching of L-arabinofuranose, D-glucuronic acid, 4-O-methyl-D-glucoronic acid at C_2 and C_3 positions of the D-xylose residue are very common. In addition, xylan from many plant species, especially those from hard woods, is acetylated mostly at C_3 position of the D-xylopyranosyl residue. The level of branching is usually dependent on the source and the developmental stage of the plant (Biely, 1985; Jeffries, 1990; Bastawde, 1992).

In the cell wall of plants cellulose microfibriles are found embedded in an aqueous gel of xylan which again is enclosed by a layer of lignin (Jeffries, 1990). Xylans form a covalent attachment with the lignin coat at various points, usually between the free carboxy group of its side chain and the benzyl ring of lignin, and interact with the underlying cellulose via hydrogen bonding (Jeffries, 1990; Kato and Nevins, 1985). This structural arrangement is believed to be important in maintaining the integrity of the cellulose and protect the fibers from attack by cellulases (Uffen, 1997).

4. Enzymes involved in the hydrolysis of xylan

Hydrolysis of xylan to its constituent sugars is brought about by an array of enzymes. The enzyme involved in the hydrolysis of the internal β-1,4-xylopyranosidic linkage is known as endo xylanase, which randomly attacks the internal β- 1,4- linkage. The main products of the reaction catalyzed by endoxylanase are xyloologosaccharides of different chain length. Xylooligosaccharides thus produced are acted upon by β-xylosidase to produce xylose. Both xylanase and β -xylosidase are not capable of hydrolysing the side chain sugars at the C_2 and C_3 position of xylose residues of xylan. This function is therefore carried out by side chain hydrolysing enzymes. These are α-glucuronidase which is involved in the hydrolysis of glucuronic acid and 4-O-methyl glucuronic acid side chains; α-arabinofuranosidase, which hydrolyses L-arabinose side chains; and acetyl xylan esterase which is involved in the deacetylation of acetylated xylan. Of all the enzymes involved in the hydrolysis of xylan, attention was mainly

focused on the study of xylanases and to some extent β-xylosidase from different sources. Up to now relatively little is known about the different debranching enzymes (Bastawde, 1992; Coughlan and Hazlewood, 1993).

5. Industrial applications of xylanases

Much of the interest on the study of xylanases has been driven by the potential application of these enzymes in different industrial processes. Though in some areas successful application is already underway (Viikari *et al.,* 1994), the search for better xylanases which can fulfil each specific need is currently in progress. The major areas of application of xylanases can be summerized as follows.

5.1. Hydrolysis of xylan from lignocellulosic waste

Annually a large amount of xylan containing waste is released in the form of industrial, agricultural, and municipal wastes. Hydrolysis of such wastes, in addition to providing fermentable sugars, offer a good alternative of waste management (Biely, 1985; Broda, 1992; Hespell *et al.*, 1997). The reducing sugar produced can be used for the production of lactic acid, ethanol, propionic acid, acetone, single cell protein, and a range of many other products (Laplace *et al.,* 1993; Olsson and Hahn-Hagedal, 1993; Ramsay *et al.,* 1998). Xylan can be easily hydrolysed using mineral acids. However, acid hydrolysis often lead to the formation of toxic side products which inhibit the growth of microorganisms in subsequent bioconversion processes (Bastawde, 1992). An alternative method is enzymatic hydrolysis which offers mild reaction conditions with no toxic side products for subsequent bioconversion.

The xylan fraction of hemicellulosic waste is found complexed with other lignocellulosic components (Jeffries, 1990). Hence, for efficient hydrolysis, besides xylanases other plant cell wall polymer degrading enzymes need to be employed as well. Moreover, because of the solubility of xylan at alkaline pH, and the alkaline nature of some xylan containing wastes, xylanses with optimum activity in the alkaline pH range could prove to be extremely important (Grant and Horikoshi 1992).

5.2. Enzyme assisted kraft pulp bleaching

The first step in the production of pulp following the kraft process is cooking of wood chips at alkaline pH, a process that release about 90-95% of the lignin and a large part of the hemicellulose component (Bajpai and Bajpai, 1992; Senior *et al.,* 1992; Daneault *et al.,* 1994; Viikari *et al.,* 1994). The remaining 5-10% lignin is highly modified under the process of cooking thus giving the characteristic black or brown color of kraft pulps. To produce high quality white paper, a series of bleaching steps are carried out to remove the residual modified lignin. Traditionally chlorine and chlorine dioxide are used as bleaching agents (Bajpai and Bajpai, 1992). Though chlorine based bleaching is effective in removing the residual lignin, a large amount of chlorinated organic compounds are produced and find their way to receiving water bodies (Daneault *et al.,* 1994; Viikari *et al.,* 1994). Some of these chlorinated organic compounds are considered to be toxic and carcinogenic (Kringstad, 1989), thus causing a lot of concern of environmental pollution. With growing public understanding of the danger of such pollution, alternative bleaching methods that can reduce the use of chlorine and thus the level of pollutants in the effluent, or totally replace chlorine with other safer methods have been sought.

Xylanases were found to be important in the process of kraft pulp bleaching (Viikari *et al.,* 1994; Shoham *et al.,* 1992). Different studies showed that xylanase treatment of kraft pulp prior to the normal bleaching step resulted in a substantial reduction in the level of chlorine bleach used in subsequent bleaching operations, with concomitant reduction in the level of chlorinated organic pollutants. It has also been shown that xylanase treatment is important in achieving the necessary brightness of pulp in totally chlorine free bleaching operations (Senior *et al.,* 1992; Daneault *et al.,* 1994; Wong *et al.,* 1997).

Because the first step in pulp bleaching operation is carried out at elevated temperature and alkaline pH, the incoming pulp for enzyme treatment has an alkaline pH and high temperature. On the other hand most xylanases known to date are active at lower temperature and in the neutral or acidic pH range. Therefore, this necessitates cooling and pH adjustment of the large pulp mass during enzyme treatment followed by re-

heating and increasing the pH for subsequent bleaching operations (Wong and Saddler, 1993). In addition to the direct cost of cooling and heating energy and the cost of acids, this operation takes time, which is unacceptable by many pulp and paper mills. To avoid this problem there is an on going search for alkaline active and thermostable xylanases that could reduce or avoid the need for temperature and pH readjustment.

5.3. Animal feed supplement

In recent years enzymes are proving extremely important as feed supplements, especially for monogastric animals. The enzymes that are found important for this application are xylanases, proteases, phytases, and amylases (Wong and Saddler, 1993; Bedford and Morgan, 1996; Bedford, 1996; Biehl and Baker, 1997; McCoy, 1998).

The most important application of xylanases in the feed industry is as supplement for chicken feed (Campbell and Bedford, 1992; Biehl and Baker, 1997). Chiken fed with xylan-rich feeds (e.g. rye, wheat and triticale) are known to face problems of reduced nutrient utilization, impairment of fat retention (the effect being very severe for long chain fatty acids), reduced feed consumption, and thus retarded growth (Antoniou and Marquardt, 1981; 1982). These effects are known to be caused due to the presence of high amount of xylan which becomes highly viscous in the intestine thus impairing the process of digestion and absorption (Campbell and Bedford, 1992). In addition, the presence of high concentrations of xylan results in a sticky litter consequently forming balls of the excreta on the toes of the chicken. Addition of crude xylanase preparation as feed supplements was shown to alleviate many of these problems (Jakisch and Jeroch, 1990; Richter *et al.*, 1990; Bedford, 1996). The pure xylanase preparation, however, was not as effective as the crude preparation indicating the important role of debranching enzymes in xylan depolymerization (Wong and Saddler, 1993; Campbell and Bedford, 1992). Different studies showed that supplementation of xylan-rich feed with xylanase does not completely eliminate the stickiness of the litter (Bedford and Morgan, 1996), which may show that no complete depolymerization takes place. This strongly indicates the need to search for xylanases having higher efficiency under the conditions of the animal gut.

5.4. Other applications

Xylanases also find potential applications in a number of industrial processes. In the food industry, xylanases along with pectinases and cellulases can be used for the maceration of fruit and vegetable materials, for the clarification of juice and wines (Felix and Vllettaz, 1983; Wong and Saddler, 1993). They are also important for the extraction of oils (Godfrey, 1983), starch (Christophersen *et al.,* 1997), and for the modification (or improvement) of textural and staling properties of baked products (Reichelt, 193; Poutanen, 1997). Another area of application for xylanases is in the production of dissolving pulps, which are purified celluloses used for making viscous rayons, cellulose esters, and cellulose ethers (Wong and Saddler, 1993). The presence of xylan in the pulp prior to derivatization creates a variety of problems. Xylanases have been used for the selective removal of xylan in the preparation of dissolving pulp (Paice and Jurasek, 1984; Christov and Prior, 1993).

6. Search for novel xylanases of potential application

Early studies on xylanases concentrated mainly on the study of fungal enzymes. In general fungal xylanases are optimally active below the neutral pH range and at or below 50°C (Coughlan *et al.,* 1993). Increase in the range of applications of xylanases in recent years led to the search for novel xylanases from many other sources with the view of developing enzymes better suited for each particular area of application.

Some xylan containing industrial wastes, such as kraft pulp effluent from the initial cooking stage, have alkaline pH. In addition xylan by its very nature is soluble at alkaline pH. As a result alkaline xylanases are considered to have a better potential for the hydrolysis of xylan. In enzyme assisted pulp bleaching operations, the use of xylanases having optimum activity at elevated temperature and alkaline pH offer tremendous technical and economic advantages (Zamost *et al.*, 1991).

Generally two approaches have been followed by researchers to obtain xylanases (and other enzymes as well) having the required property (Janecek, 1993). These are: 1) isolation of new strains from the environment producing enzymes of better potential, and/or 2) engineering of existing enzymes (from mesophilic and neutralophilic strains)

through protein engineering. Though impressive progress has been made in recent years in developing and perfecting protein engineering techniques through site directed mutagenesis, our understanding of protein structure to function relationship is not still perfect. Predictions made on the basis of structural information have in many occasions failed to bring about the desired result. Another version of protein engineering is random mutagenesis (or as some call it directed evolution). This basically mimics what is happening in nature, except that it is carried out in vitro in a very short time (Moore and Arnod, 1996; Kuchner and Arnod, 1997). Nevertheless considering the number of clones one needs to screen to get the desired change (especially when changes are desired at many positions) makes it difficult and cumbersome (Kuchner and Arnod, 1997).

Thus isolation of new strains producing novel enzymes of potential industrial importance from the environment does offer the best option. This is especially important when one considers the fact that upto now about 1% or less of the microorganisms that are believed to exist on earth are known, and far less than this is deposited in culture collections world wide (Colwell, 1997). In this regard extreme environments which are characterized with high temperature, very alkaline or acid pH, etc. are expected to offer novel strains producing xylanases of tremendous potential for different industrial applications. These environments can be considered as places where nature has been 'doing protein engineering' over thousands and millions of years.

Though mesophilic microbial strains with optimum growth in the neutral pH range are known to produce alkaline active proteases and very thermostable amylases, to date only few thermostable (Mitsuishi *et al.*, 1987; Breccia *et al.*, 1998) and no alkaline xylanases has been reported from such organisms. To obtain thermostable xylanases (and other thermostable enzymes as well) many researchers focused their attention on the isolation of thermophilic microoorganisms. As a result many xylanase producing thermophilic fungi, bacteria, and archea have been isolated in different laboratories (Winterhalter and Lieb 1995; Sunna and Anthranikian, 1996; Sunna *et al.*, 1996; Breccia *et al.*, 1998). Though most of these organisms produce thermostable xylanases,

all the enzymes showed optimum activity and stability at or below neutrality (Zamost *et al., 1991*; Sunna and Anthranikian, 1996).

Other studies were directed to the study of xylanases from alkaliphilic microorganisms with the expectation that extracellular enzymes from such organisms would be adapted to be optimally active and stable at alkaline pH (Grant *et al.,* 1990; Horikoshi, 1996). Over the last two decades a number of xylanolytic alkaliphilic and alkalitolerant strains were isolated from different parts of the world. However, contrary to the expectation, most of the xylanases from these organisms showed optimum activity around neutrality, though some of them retain appreciable activity in the alkaline pH range (Tsujibo *et al.,* 1990; Blanco *et al.,* 1995; Dey *et al.,* 1992; Kang *et al.,* 1996; Okazaki *et al.,* 1985; Park *et al.,* 1992; Ratto *et al.,* 1992; Nakamura *et al.,* 1993). Moreover, the great majority of xylanases derived from alkaliphiles and alkalitolerant microorganisms are optimally active and stable at or bellow the temperature range of 50-55°C (Tsujibo *et al.,* 1990; ; Nakamura *et al.,* 1993; Horikoshi,1996).

Almost all xylanolytic alkaliphiles known so far were isolated from neutral soil. As stated above (Section 2.1), neutral and even acidic soil samples, harbour obligate and facultative alkaliphiles, which are supposed to live in alkaline microenvironments generated by some biological activities. And almost all the strains belong to the genus *Bacillus*. The question whether these alkaliphilic bacilli evolved (mutated) from neutralophilic strains to occupy these microenvironments in the course of competition for survival or represent a separate evolution remains to be answered. The extent of alkalinity of these (micro)habitats and the extent of alkalinity to which their extracellular enzymes are exposed to and the selective pressure for alkaline active enzymes is not yet determined. All the evidence available so far show that the vast majority of alkaliphiles isolated from soil produce xylanases active around neutrality, though few of them retain appreciable activity in the alkaline pH range.

Another source of alkaliphiles, hitherto over looked, is isolation of xylanolytic alkaliphiles from naturally occurring alkaline habitats. In our laboatory we isolated different alkaliphiles producing alkaline proteases, amylases, cellulases, etc. of

potential industrial application from alkaline soda lakes in Ethiopia (Gessesse and Gashe, 1997; Gessesse, 1998). This may show the great potential of these environments for the isolation of novel microbial strains producing enzymes of potential biotchnological importance.

However, xylanases from new microbial strains may not fulfil all the requirements for a particular area of application. The selective pressure microorganisms face in their respective habitats and their enzymes evolve is different from the requirement for industry. In order to get enzymes of potential industrial application (xylanases and other enzymes) it may probably be worthwhile to combine screening of new strains from as diverse habitats as possible and fine tune such enzymes using protein engineering.

7. Methods of enzyme production

Up to now almost all commercial enzymes are produced through submerged fermentation in large volume followed by a series of downstream processing. With increasing application of xylanases and other enzymes the existing enzyme production and distribution methods are increasingly viewed as expensive which could potentially limit its wide spread application. This is especially so when one considers application of xylanases and other enzymes in the animal feed industry where a large quantity of feed enzyme is required. One alternative currently pursued by some industries is to clone and express the xylanase gene in plants (Pen *et al.*, 1993; Herbers *et al.*, 1995; McCoy, 1998). The seeds derived from these transgenic plants can then be used as animal feed. Extraction of the enzyme from the seed and applying it to the feed could be another alternative. In light of the observation that pure xylanases are not as effective as the crude preparation (Campbell and Bedford, 1992), this method will call the simultaneous expression of the debranching enzymes together with the xylanase gene.

Finding other ways of producing xylanases by the microorganisms themselves is a worthwhile pursuit. In this study two potentially useful methods, immobilization and solid state fermentation were studied for the production of xylanase.

7.1. Immobilization

To make the production of microbial enzymes cost effective it is considered important to increase product yield through the use of high cell density in the fermentation process. One way to do this is cell immobilization (Mattiasson, 1983a,b; Kiy and Tiedtke, 1992). The use of immobilized cells for different biotechnological processes offer several advantages (Mattiasson, 1983a,b; Brodelius and Vandamme, 1987; Rehm and Omar, 1993; Tonkova *et al.*, 1994; Tanaka *et al.*, 1994). For example some of these advantages are: prolonged metabolic activity of the organisms and ability to repeatedly produce the product of interest; better tolerance against poisonous substances/ or inhibitors; reduced risk of contamination; ability to carry out continuous fermentation with less sophisticated reactors.

Different species of fungi and bacteria have been immobilized for the production of extracellular enzymes (Singh *et al.*, 1990; Federici *et al.*, 1990; Kanasawud *et al.*, 1989; Kanasawud *et al.*, 1992; Tonkova *et al.*, 1994; Kang *et al.*, 1995; Mamo and Gessesse, 1997). In many organisms production of extracellular enzymes by immobilized cells have been shown to give higher yield than freely suspended cells (Shinmyo *et al.*, 1982; Dobreva *et al.*, 1996). One reason could be that, in addition to the high cell density employed, immobilization reduce the effect of catabolite repression of enzyme synthesis. This is important when immobilized cells are used under continuous systems. Moreover, enzyme production by immobilized cells is expected to make the production process simplified and reduce the over all production cost (Mamo, 1996).

Upon immobilization, cells are subjected to a changed environment which could result in alteration of their physiology. For example, immobilized *Bacillus amyloliquefaciens* showed a 50% reduction in its respiratory activity and more than 80% reduction in its growth rate (Shinmyo *et al.*, 1982). A similar reduction in growth rate and metabolic activity as well as a change in morphology upon immobilization was observed in many other cell types (Rehm and Omar, 1993; Kiy and Tiedtke, 1993; Muller *et al.*, 1994). One major effect of immobilization which result in a change in the physiology of the

organisms is a reduction in oxygen transfer (Mattiasson, 1983 a, b; Omar, 1993). In free cell culture, cells obtain oxygen from the surrounding liquid. Immobilization imposes a barrier for the diffusion of oxygen, where only cells at the outer region get enough oxygen. In addition the transfer of other substances is also greatly altered leading to a change in the physiology of immobilized cells.

Methods commonly used for cell immobilization are: entrapment, adsorption, crosslinking (covalent coupling), affinity immobilization, etc. (Mattiasson, 1983a,b; Brodelius and Vandamme, 1987). Of all these methods, immobilization by entrapment is the most commonly used method. Immobilization by entrapment is carried out using natural polymers, such as alginate, agar, κ-karageneen, or using synthetic polymers such as polyacrylamide and polyurethane.

To date many different types of cells, including aerobic and anaerobic thermophiles (Klingberg *et al.,* 1990; Mamo and Gessesse, 1997) have been immobilized for the production of extacellular enzymes. However, inspite of their potential usefulness, upto now there is no report on the production of enzymes by immobilized alkaliphilic microorganims.

7.2. Solid state fermentation

Since the advent of the antibiotic era around the period of the second world war, cultivation of microorganisms using submerged culture became popular. The different parameters that affect the growth of microorganisms in submerged culture have been well understood. Different reactors allowing improved cultivation have been designed together with improvement on different control (monitoring) systems. On the other hand cultivation of organisms using solid state fermentation, once popular, was almost abandoned in the developed nations. However, in many Asian countries solid state fermentation has been in use for thousands of years and still is popular for many applications (Pandy, 1992).

Solid state fermentation (SSF) is defined as the growth or cultivation of organisms in the absence of free-flowing water. However, the substrate should contain sufficient

moisture to support growth and metabolism. Compared to submerged fermentation (SMF), SSF offers many advantages (Tobey and Yousten, 1976; Lonsane and Ramesh, 1990). The main ones are: complex machinery, equipment, and control systems are not required; the media used is simple and cheap; there is reduced energy demand; usually high concentration of the product is produced; easier scale up of the process; and absence of foam build-up. The risk of contamination is lower than SMF due mainly to the inability of many organisms to grow at low water activity. In addition, in the case of SSF the product can be extracted using less volume of liquid (Ramakarishna *et al.*, 1982; Lonsane and Ramesh, 1990). It has been shown that in most cases the same amount of product is found in at least 10 fold less volume of liquid than it is in SMF fermentation broth. Therefore, using SSF up to 10-fold less volume of liquid is handled. This offers a number of advantages. First, less volume to handle means less volume to dispose. In the fermentation industry, disposal of large volume of liquid after appropriate waste treatment operations is becoming a real challenge (Hahn-Hagerdal, 1986). Hence, the use of SSF could allow to cut down waste treatment costs by about 10 fold. Less volume of liquid to handle has also an advantage during downstream processing. In the case of SMF the cell free culture supernatant need to be concentrated before recovery of the enzyme through precipitation or other means. This, in addition to being energy intensive and time consuming, could lead to denaturation (product loss). Less volume of liquid in the case of SSF could allow direct precipitation of the product (Datar, 1986). It is also possible to dry the SSF culture, powder it and use it for subsequent application, which could improve enzyme storage.

Some of the limitations of SSF are: that it is labour intensive; lack of uniformity in the substrate; due to the requirement of many organisms for high water activity there is a limited choice on the type of organisms that can grow using SSF culture (Lonsane and Ramesh, 1990; Pandey, 1992).

Generally fungi have been found more suitable for SSF than bacteria, which mostly need higher water activity (Lonsane and Ramesh, 1990). As a result, up to now most studies on SSF were made using fungi. However, some strains belonging to the genus *Bacillus* have been shown to produce high level of amylase (Ramesh and Lonsane,

1989) and xylanase (Archana and Satyanarayana, 1997) activity when grown using solid state fermentation.

8. Objectives of the study

In Ethiopia there are many productive alkaline soda lakes which are found along the Great Rift Valley of East Africa. The lakes are expected to support a diverse group of heterotrophic bacterial communities some of which are involved in the degradation of biological polymers. The objectives of the present study were to isolate xylanase producing alkaliphilic bacteria from one of these alkaline soda lakes, purify and characterize the enzymes to determine potential applications, and investigate methods of enzyme production that would allow the cheap production of the enzyme(s).

II. MATERIALS AND METHODS

1. The organisms and cultivation conditions

1.1. Isolation

Water and sediment samples were collected from Lake Arenguadie, an alkaline soda lake in Central Ethiopia using sterile plastic tubes. A loop full of the sample was streaked on nutrient agar plates, pH adjusted to about 10.3 by adding sterile sodium carbonate solution to a final concentration of 1% (w/v) after autoclaving. After 48 h incubation at 32°C, individual colonies were picked and transferred to fresh nutrient agar plates.

1.2. Screening for xylanase production

All isolates were screened for xylanase production by growing them on xylan medium consisting of (g/l): xylan, 5; peptone, 5; yeast extract, 2; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; and Na_2CO_3, 10. For solid media 15 g/l purified agar was added. Sodium carbonate was sterilized separately and added to the rest of the medium after cooling. After 48 h incubation the plates were flooded with 0.1 % aqueous Congo red solution and washed with 1 M NaCl (Teather and Woo, 1982). Xylanase producing colonies form a clear hallow around the colony. To select the best enzyme producing strains all isolates that were positive for xylanase production on xylan agar plates were grown in liquid culture (50 ml xylan medium in 250 ml flask) and incubated with rotary shaking at 32°C. The level of xylanase production was determined from the cell free culture supernatant.

1.3. Enzyme production in liquid culture

One hundred ml xylan medium in 500 ml baffled flasks was inoculated with 2 ml of a 24 h culture and incubated with rotary shaking at 32°C. The cell free supernatant obtained after centrifugation was used as the enzyme source.

2. Enzyme purification

The cell free culture supernatant was precipitated using solid ammonium sulphate to 70% saturation. After centrifugation the pellet was dissolved in 10 mM Tris-HCl buffer, pH 8, and dialysed against 3 changes of the same buffer. The dialysed enzyme

was applied to DEAE-Sepharose column (2.5 x 12 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8. After the enzyme was applied the column was eluted first with buffer alone followed by a linear gradient of 0 to 0.5 M NaCl at a flow rate of 90 ml/h. Fractions containing xylanase activity were pooled, concentrated, and dialysed against 10 mM Tris-HCl buffer, pH 8 .The concentrated enzyme preparation was applied to Sephadex G-75 column (1.5 x 110 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8 and eluted at a flow rate of 12 ml /h. In the case of AR-009 xylanase fractions containing xylanase activity were pooled, concentrated, and reapplied to Sephadex G-75 column and eluted as above.

3. Enzyme production through cell immobilization

3.1. Cell immobilization

Cells were immobilized in agar beads following the methods of Nilsson *et al.*, (1983). Four g (wet weight) of cells was suspended in 40 ml of 3% agar solution and beads were formed by dropping the agar-cell slurry to a layer of vegetable oil. After thorough washing with sterile saline solution, beads were transferred to fresh xylan medium and incubated at 37°C with rotary shaking.

3.2. Continuous xylanase production

Continuous xylanase production was carried out in a jacketed glass column with a height of 20 cm and internal diameter of 4 cm. The experimental set up used for continuous cultivation is shown in Fig. 1. The temperature was kept constant at 37°C by continuously circulating water through the jacketed column from a thermostat controlled water bath. Diluted xylan medium (1:4 diluted with the concentration of Na_2CO_3 maintained at 1%) was continuously pumped at a dilution rate of 0.39/h. The reactor was aerated by continuous pumping of filtered air at a rate of 80 cc/min.

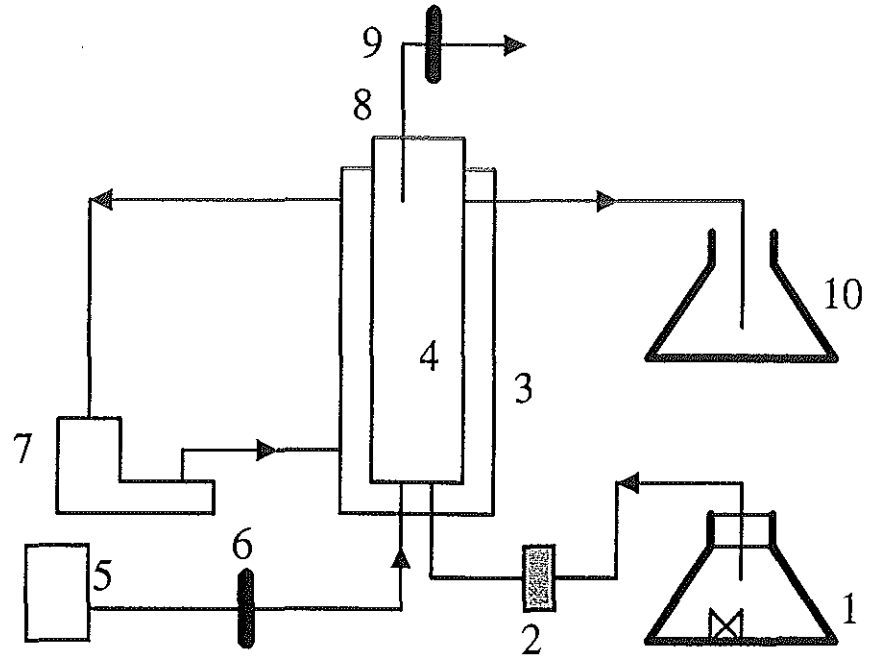


Fig 1. Experimental set-up used for the continuous production of xylanase by immobilized cells of *Bacillus* sp. AR-009. 1, feed tank with continuous stirring; 2, peristaltic pump; 3, jacketed column; 4, reactor column with immobilized cells; 5, aerator; 6, filter; 7, thermostated water bath; 8, out let for exhaust gases; 9, filter; 10, effluent

4. Solid state fermentation (SSF)

4.1. *Xylanase production using solid state fermentation*

To 10 g of wheat bran in 250 ml Erlenmeyer flask, stock mineral salt solutions were added to give a final salt concentration of (mg/g): K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2; and $CaCl_2.2H_2O$, 0.1. Distilled water was added in such a way that the final wheat bran to moisture ratio was 1:1.5 (w/w). After sterilisation by autoclaving, the flasks were cooled and sterile Na_2CO_3 solution was added to give a final concentration of 10% (w/w). The flasks were inoculated with 10% inoculum (v/w) of an overnight culture and incubated at 37°C for 72 h.

4.2. Effect of moisture level

The effect of moisture level on xylanase production was tested by varying the wheat bran to moisture ratio in the range of 1:0.5 to 1:4.5 (w/w). All the liquid added in to the flask was taken into consideration in calculating the wheat bran to moisture ratio.

5.3. Assay for protease activity

Protease activity was assayed at 50°C as described earlier (Gessesse and Gashe, 1997). One unit of protease activity was defined as the amount of enzyme which resulted in the release of 1 μg amino acid equivalent to tyrosine per minute. Protease production was expressed as U/g dry bacterial bran.

5.4. Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12% gel (Laemmli, 1970). After electrophoresis the gel was stained using Coomassie brilliant blue.

5.5. Measurement of protein concentration

Protein concentration was determined using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985) using bovine serum albumin as a standard.

5.6. Thin layer chromatography (TLC)

For the chromatographic analysis of reaction products, 4 ml of 10 mg/ml oat spelt xylan dissolved in glycine NaOH buffer, pH 9, was mixed with 1 ml xylanase (5 U) and incubated at 60°C. The hydrolysate was applied on to silica gel 60 F_{254} (Merck) and developed using butane-1-ol/ethanol/water mixture (5:3:2 v/v) in 4 ascents. To develop spots the plate was sprayed with 30% (v/v) H_2SO_4 and heated at 110°C.

III. RESULTS

1. The organisms

1.1. Isolation and characterisation

A total of 200 aerobic alkaliphiles were isolated from Lake Arenguadie, an alkaline soda lake in the Rift Valley area of Ethiopia. All strains were screened for xylanase production on xylan containing solid and liquid media. Forty four strains gave detectable clear zone on solid media (Table 1). All these strains were grown in liquid xylan medium and the level of xylanase activity was determined from the cell free culture supernatant. A total of 17 isolates produced appreciable xylanase activity in liquid culture, out of which 11 produced xylanase activity greater than 1 U/ml. Three strains, designated as AR-009, AR-022, and AR-135 were selected for further study.

Table 1. A summary of results of xylanase screening on xylan containing solid media.

Group	Xylanase production	Number of strains
1	+++	5
2	++	30
3	+	9
4	+/-	5
5	-	152

1.2. Identification of the strains

The three strains were identified to the genus level based on morphological and biochemical characteristics following *Bergey's Manual of Systematic Bacteriology* (Sneath, 1986).

1.2.1. Strain AR-009. Some biochemical and morphological characteristics of strain AR-009 is given in Table 2. The organism is a Gram-positive motile rod, with a terminal endospore. It is catalase positive, aerobic, and negative for Vogus Proskaur test. It hydrolyzed starch, casein, gelatine, Tween 80 and cellulose. It grew in the pH

range of 8-11 with no detectable growth at pH 7. On the basis of these characteristics it was identified as a strain of the genus *Bacillus*.

1.2.2. Strain AR-135. Strain AR-135 was a Gram-negative cocci, non-motile, aerobic, catalase positive, and negative for Vogus Proskaur reaction (Table 2). It grew well in the pH range of 8 to 11, but does not grow at pH 7. It forms a distinct clearing zone on xylan containing nutrient agar plates, but does not hydrolyze gelatin, casein, starch, Tween 80, and cellulose. No acid and gas formation was detected from glucose. Colonies were deep yellow in color on nutrient agar plates. On the basis of these properties the strain was identified as a species of the genus *Micrococcus*.

1.2.3. Strain AR-22-1. AR-22-1 cells are aerobic, Gram-positive, rod shaped, motile, and catalase positive. Colonies appeared golden yellow when grown using nutrient agar plates. It grew well from pH 8 to 11 and no detectable growth was observed at pH 7. No endospore was detected at different stages of growth. Identification of this strain to a specific genus proved to be difficult and await further taxonomic characterization. However, most of its characteristics suggest that it is related to the genus *Cellulomonas*.

1. 2. Time course of growth and enzyme production

The time course of growth and enzyme production was determined in a medium containing birchwood xylan as a carbon source. Xylanase production by all the strains was growth associated. For AR-009 maximum xylanase production was observed after 20 h and productivity remained more or less the same up to 56 h while biomass started to gradually decline after 40 h ((Fig. 2). Xylanase production by strain AR-22-1 reached to a maximum after 10 h and remained unaffected up to 56 h (Fig. 3). A similar pattern was also observed for strain AR-135 with maximum activity attained after 24 h (data not shown).

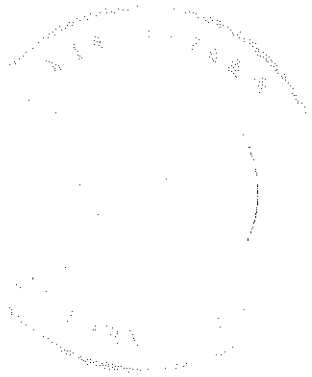


Table 2. Morphological and biochemical characteristics of three xylanase positive strains

Characteristics	Strain		
	AR-009	AR-22-1	AR-135
Gram stain	positive	positive	positive
Morphology	rod	rod	coccus
Endospores	present	absent	absent
Sporangia	swollen	---	------
Pigment	white	golden	yellow
Motility	motile	motile	nonmotile
Hydrolysis of:			
Starch	positive	positive	negative
Casein	positive	positive	negative
Gelatine	positive	positive	negative
Tween 80	negative	negative	negative
Cellulose	positive	positive	negative
V.P reaction	negative	negative	negative
Catalase	positive(weak)	positive	positive
Growth in nutrient agar containing NaCl:			
0.5%	positive	positive	positive
20%	positive	positive	positive
25%	negative	negative	negative
Gas from glucose	negative	negative	negative
Acid from glucose	positive	negative	negative
Growth on nutrient agar at:			
pH 7	negative	negative	negative
pH 8	weak	weak	positive
pH 10	positive	positive	positive
pH 11	positive	positive	positive

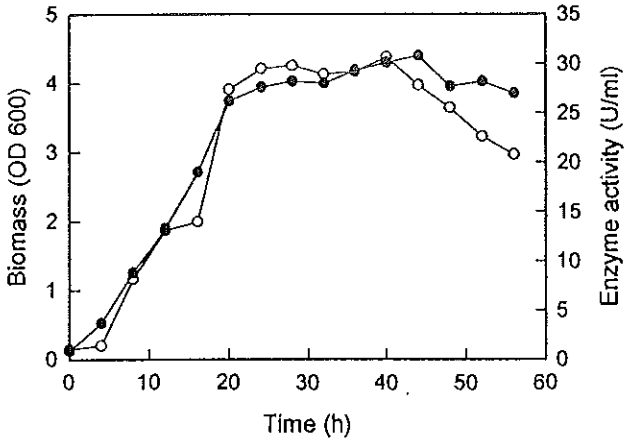


Fig 2. Time course of growth and enzyme production by AR-009. ○, Biomass, OD at 600 nm; ●, xylanase production (U/ml).

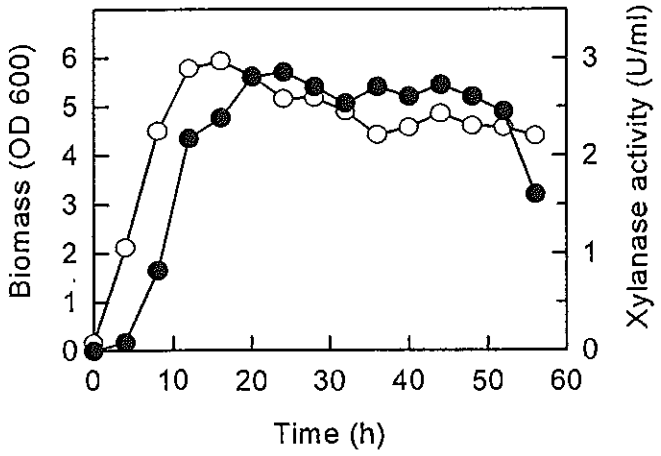


Fig. 3. Time course of growth and enzyme production by AR-22-1. ○, Biomass, OD at 600 nm;, ●, xylanase production (U/ml).

2. Effect of different carbon sources on enzyme production

The effect of different carbon sources on the level of xylanase production by the 3 strains is presented in Table 3. AR-009 produced high level of xylanase activity when grown in media containing oat spelt xylan, wheat bran and birch wood xylan as carbon sources. In addition appreciable xylanase activity was detected when starch, sucrose, arabinose, glucose, and xylose were used as carbon sources. However, compared to xylan and wheat bran, these sugars induced low level of xylanase activity, the lowest being in the presence of xylose. This may indicate that that xylose is a repressor of xylanase synthesis by *Bacillus* sp. AR-009. One interesting property of AR-009 worth noting is that the level of xylanase production is much higher than any other xylanolytic bacterial strains known to date.

Table 3. Effect of different carbon sources on xylanase production.

Sugar	AR-009	AR-135	AR-22-1
Oat spelt xylan	45.2	2.5	2.8
Birchwood xylan	35.0	2.7	2.7
Wheat bran	44.6	ND	1.6
Starch	21.0	NA	ND
Sucrose	14.6	NA	0
Arabinose	14.1	NA	1.1
Glucose	10.6	NA	0
Xylose	6.0	3.7	2.3

NA: no activity; ND: not done

Xylanase production by AR-135 was also induced in the presence of xylan and xylose. A better xylanase production was observed in the presence of xylose than in the presence of xylan. Only 73% of the xylanase produced in the presence of xylose was detected when the organism was grown using xylan (Table 3). Another difference with strain AR-009 observed was that no extracellular xylanase activity was detected when strain AR-135 was grown in the presence of other carbohydrate sources.

Xylanase production by strain AR-22-1 was induced when xylan and hydrolysis products of xylan (xylose and arabinose) were used as carbon sources. The level of xylanase produced in the presence of xylose was 82% of that produced in the presence of xylan. No xylanase activity was detected when the organism was grown in the presence of glucose, sucrose and lactose. In most other xylanolytic microorganisms, xylose is known to repress xylanase synthesis.

3. Purification and characterization of the enzymes

3.1. Bacillus sp. AR-009 xylanase

3.1.1. Purification of the enzymes

Two xylanases, designated as xylA and xylB, were purified from the culture filtrate of *Bacillus* sp. AR-009 following ammonium sulphate fractionation, ion exchange, and gel filtration chromatography. Of the two enzymes only xylB was adsorbed to DEAE-Sepharose at pH 8 and eluted using a linear gradient of NaCl.

The result of the purification is summarised in Table 4. XylA and xylB appeared homogenous on SDS-PAGE and their molecular weight was estimated to be 23 and 48 kD, respectively (Fig. 5a). The activity of the two enzymes recovered after the final purification step was different.

Table 4. Summary of the purification procedure of the two xylanases from the cell free culture supernatant of Bacillus sp. AR-009.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Culture filtrate	67243	1729.0	38.3	1.0	100.0
Ammonium sulphate	51342	322.0	159.4	4.1	76.4
Ion Exchange					
Xyl A	46490	126.5	367.5	9.4	69.1
Xyl B	1109	14.0	79.2	2.0	1.7
Gel filtration (1st)					
Xyl A	14032	30.6	458.6	11.8	20.9
Xyl B	605	6.0	100.8	2.6	0.9
Gel filtration (2nd)					
Xyl A	9174	17.5	524.2	13.5	13.6
Xyl B	90	1.1	367.9	9.5	0.6

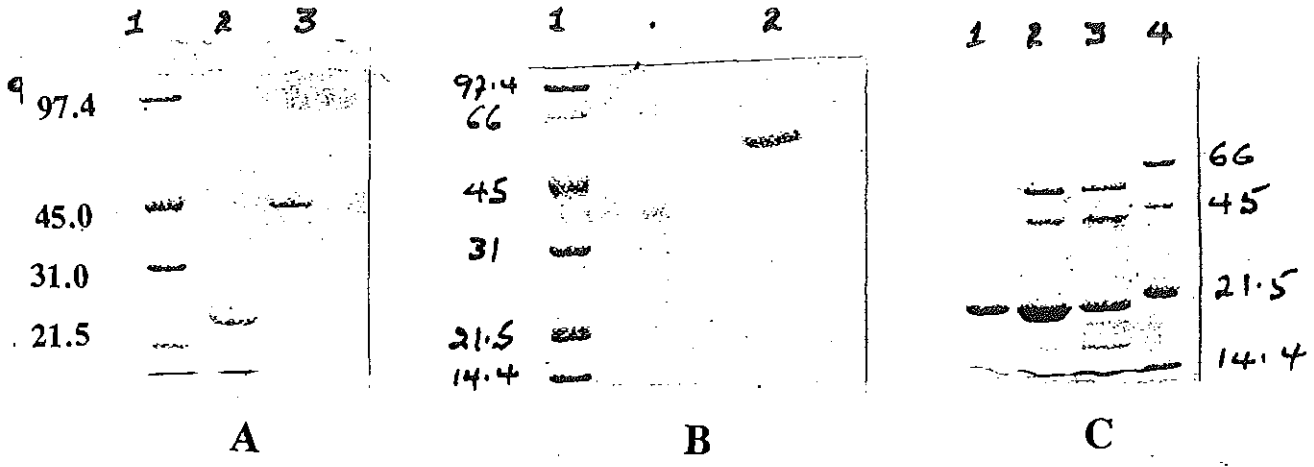


Fig. 4 . SDS-PAGE of the purified enzymes from the three strains. **A.** *Bacillus* sp. AR-009 xylanases: lane 1, molecular weight markers; lane 2, xylA; and lane 3, xylB. **B.** *Micrococcus* sp. AR-135 xylanase: lane 1, molecular weight markers; lane 2, AR-135 xylanase. **C.** strain AR-22-1 xylanase: lane 1, AR-22-1 xylanase after gel filtration; lane 2, after ion exchange chromatography; lane 3, crude enzyme preparation; and lane 4, molecular weight markers.

3.1.2. Effect of temperature on activity and stability of xylA and xylB. The effect of temperature on activity was determined at different temperature values using pH 8 and pH 9 buffers. The result is presented in Fig. 5. At pH 8, xylA showed optimum activity at 70°C while at pH 9 its optimum was shifted to 60°C. The optimum temperature for the activity of xylB was 70°C at pH 8 and 75°C at pH 9. The effect of temperature on stability was determined by incubating each enzyme at 60 and 65°C using pH 8 and 9 buffer. The two enzymes showed good stability at both temperature and pH values (Fig. 6). After 3 h incubation at 60°C, xylA retained more than 95% of its original activity at both pH values. At 65°C it retained 78 and 55% of its original activity at pH 8 and pH 9, respectively. Xyl B showed better stability at pH 9 than at pH 8. At 60° it retained 51% and 74% of its original activity after 3 h incubation at pH 8 and 9, respectively. At 65°C over 54 and 67% of its original activity was retained

after 1 h incubation at pH 8 and 9 respectively.

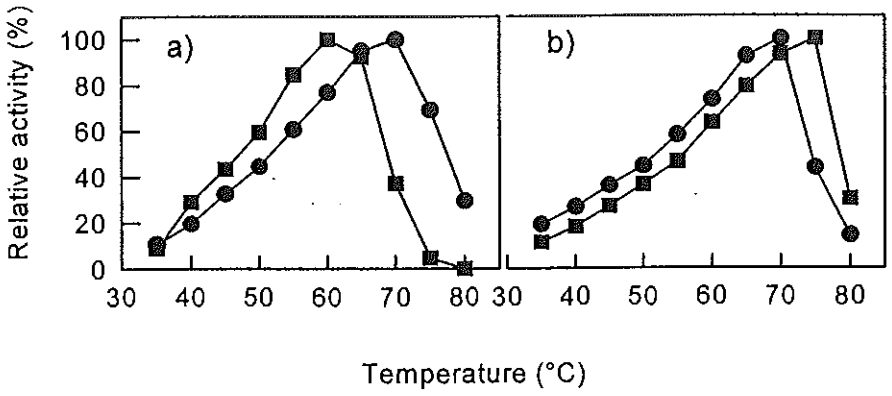


Fig. 5. Temperature profile of xylA (a) and xylB (b) of *Bacillus* sp. AR-009 assayed at pH 8 (●) or 9 (■).

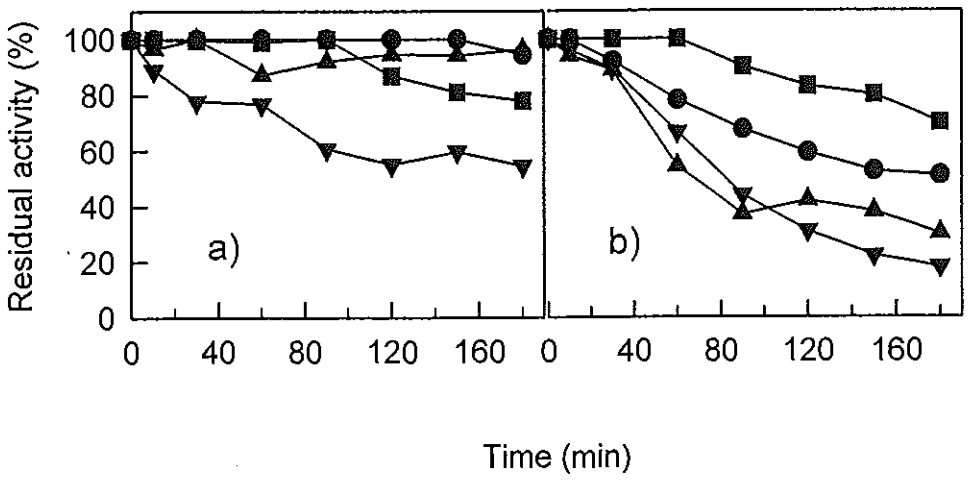


Fig. 6. Thermal stability of xyl A (a) and xyl B (b) of *Bacillus* sp. AR-009 tested at 60°C (●, ■) or 65°C (▲, ▼) and a pH of 8 (●, ▲) or 9 (■, ▼).

3.1.2. Effect of pH on activity and stability. The effect of pH on xylanase activity was determined in a range of buffers of varying pH values at 50°C. XylA was optimally active at pH 9 while xyl B was active in a broad pH range with an optimum at pH 9 to 10 (Fig. 7). The effect of pH on stability was tested by incubating the enzyme at 50°C for 1 h in different buffers of varying pH values and residual activity was measured following standard assay procedure. Both enzymes retained full activity in a broad pH range (Fig. 8).

3.1.3. Effect of metal ions on activity. Table 5 shows the activity of the two xylanases assayed in the presence of different metal ions. Both enzymes were inhibited in the presence of Hg^{2+}, Fe^{3+} Fe^{2+} and Pb^{2+}. Partial inhibition of activity was observed in the presence of Sn^{2+} for xylA and Mn^{2+} for xylB.

Table 5. Effect of different metal ions on the acivity of the two xylanases from *Bacillus* sp. AR-009. Data give as relative activity.

Metal ion (1 mM)	Xyl A	Xyl B
None	100	100
NaCl	100	103
KCl	105	105
$CaCl_2$	108	105
$MgCl_2$	99	104
$MgSO_4$	101	90
$CuSO_4$	106	95
$CoCl_2$	90	88
$MnCl_2$	74	68
$ZnSO_4$	101	105
$Pb(CH3COO^-)_2$	8	8
$FeCl_3$	0	0
$FeSO_4$	14	9
$AlCl_3$	87	92
$SnCl_2$	85	65
$HgCl_2$	0	0

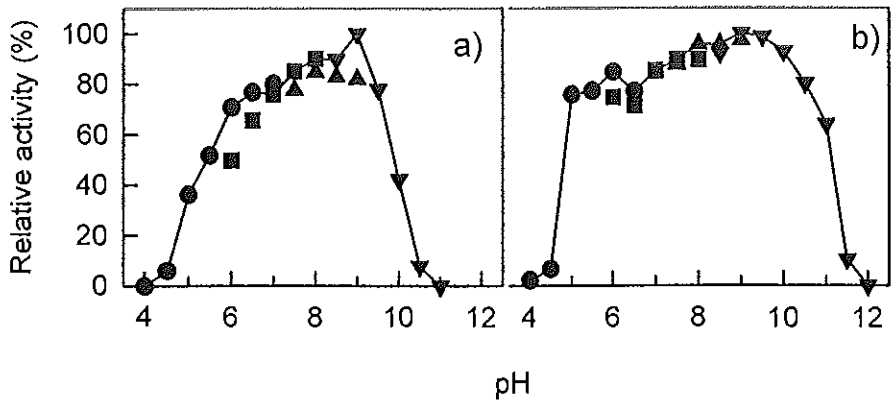
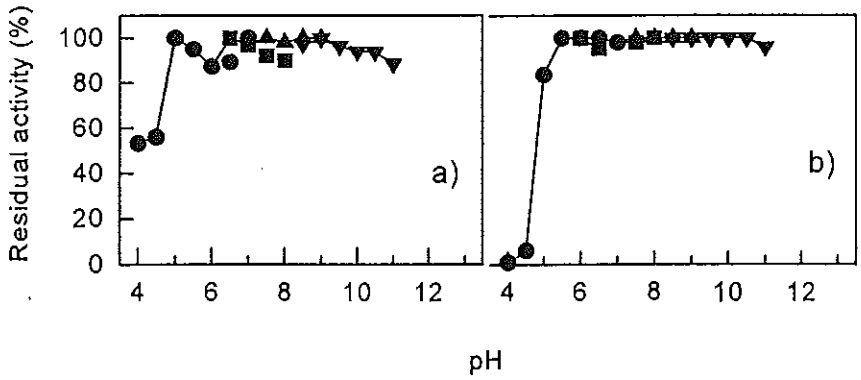


Fig. 7. pH profile of xylA (a) and xylB (b) of *Bacillus* sp. AR-009 determined at 50°C. Buffers used were: citrate-phosphate (●), phosphate (■), Tris-HCl (▲),and glycineNaOH (▼)



(▼).

Fig. 8. Effect of pH on the stability of xyl A (a) and xyl B (b) of *Bacillus* sp. AR-009. The enzyme was diluted in different buffers of varying pH values and incubated at 50°C for 1 h followed by measurement of residual activity. Buffers used were: citrate-phosphate (●), phosphate (■), Tris-HCl (▲), and glycine NaOH (▼).

3.1.4. Mode of action. The mode of action of the two enzymes was determined by measuring the rate of reducing sugar formation and viscosity reduction of oat spelt xylan following the method of Khasin *et al.*, (1993). Both enzymes resulted in a rapid reduction of viscosity and a corresponding rapid rise in reducing sugar level suggesting that they are endoxylanases (Fig. 9). Analysis of the reaction products using TLC showed the formation of a range of oligosacchrides by both enzymes (data not shown) further indicating that they are endo acting.

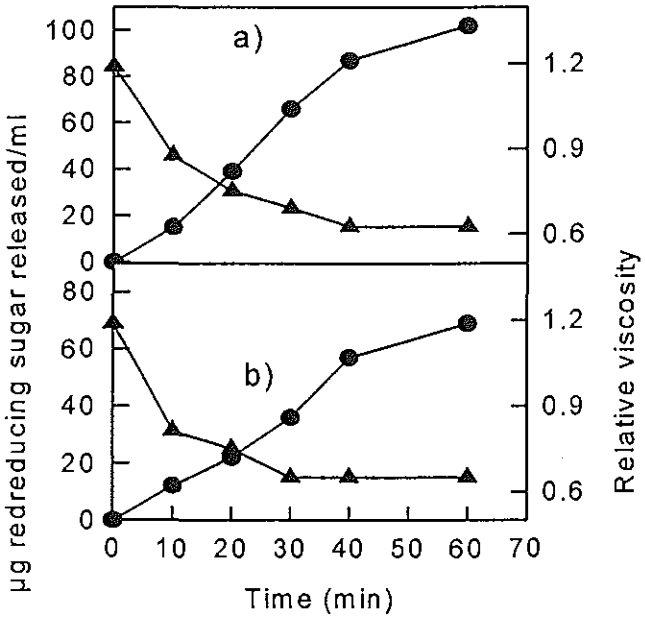


Fig. 9. Viscosity reduction (▲) and reducing sugar formation (●) from oat spelt xylan by xylA (a) and xylB (b). Oat spelt xylan (0.5%) in glycine NaOH buffer pH 9 was mixed with xylanase and incubated at 50°C. Viscosity reduction was measured using Oswald viscometer and the amount reducing sugar was determined using the DNS method.

3.2. Microococcus sp. AR-135

3.2.1. Purification of the enzyme. AR-135 xylanase was purified after ammonium sulfate fractionation, DEAE-Sepharose ion exchange chromatography and Sephadex G-75 gel filtration chromatography. The result of the purification is summarized in Table

6. The purified xylanase preparation was homogenous on SDS-PAGE (Fig. 4b) and its molecular weight was estimated to be 56 kD.

Table 6. Purification of xylanase from alkaliphilic *Micrococcus* sp. AR-135.

	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Culture filtrate	738.7	1511.4	0.49	1.0	100
Ammonium sulfate	554.9	324.7	1.7	3.5	75
Ion exchange	500.0	13.5	42.5	86.7	68
Gel filtration	368.0	3.3	111.7	228.0	50

3.2.2. Effect of temperature on activity and stability

The optimum temperature for xylanase activity was determined by varying the reaction temperature at pH 8. The enzyme was optimally active at 55°C (Fig. 10a). Thermal stability of AR-135 xylanase was determined by heating the purified enzyme at different temperature in 50 mM Tris-HCl buffer, pH 8. The data presented in Fig. 10b shows that up to 40°C the enzyme was very stable followed by a rapid lose of activity above 45°C.

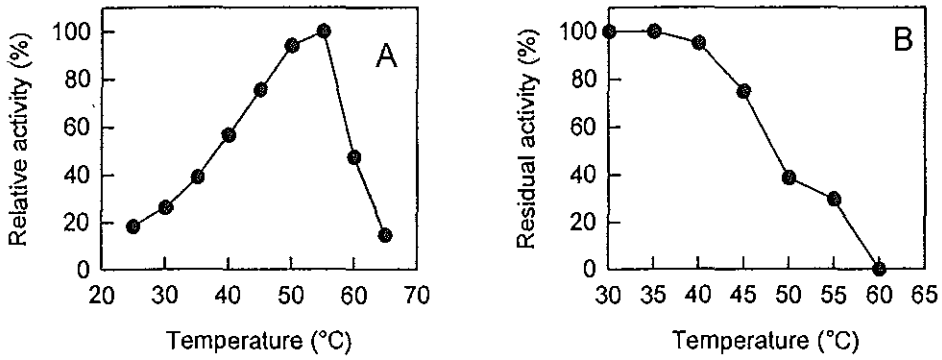


Fig. 10. Effect of temperature on activity (A) and stability (B) of AR-135 xylanase. The temperature profile was determined by assaying enzyme activity at different temperature values using 50 mM Tris-HCl buffer, pH 8. Effect of temperature on stability was tested by incubating the enzyme at different temperature values for 15 min in 50 mM Tris-HCl buffer pH 8 and the residual activity was assayed at 40°C.

3.2.3. Effect of pH on activity and stability. The pH profile of the enzyme was determined using different buffers of varying pH values. The enzyme was active in a broad pH range with an optimum at pH 7.5 to 9.0 (Fig. 11a). At pH 11, 60% of the maximum activity was displayed. The effect of pH on stability, determined using different buffers of varying pH values at 40°C, is shown in Fig. 11B. The enzyme was stable in the pH range of 6.5 to 10.

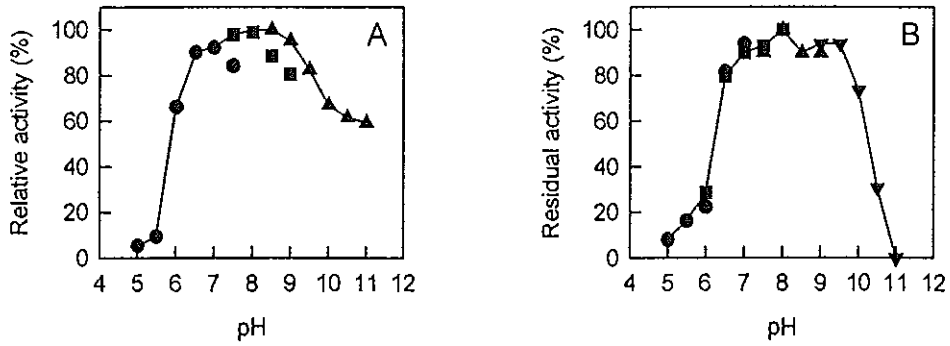


Fig. 11. Effect of pH on activity (A) and stability (B) of AR-135 xylanase. The pH profile was determined in different buffers of varying pH values at 40°C. The buffers used were: citrate-phosphate (●), Tris-HCl (■), and glycine-NaOH (▲). The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 30 min at 40°C and the residual activity assayed following standard assay procedure. Buffers used were: citrate-phosphate (●), phosphate (■), Tris-HCl (▲), and glycine-NaOH (▼).

3.2.4. Effect of metal ions on activity. The effects of different metal ions on AR-135 xylanase was tested by assaying enzyme activity in the presence of different metal ions. Strong inhibition was observed in the presence of Hg^{2+} and Cu^{2+} while Fe^{3+} Pb^{+}, and Zn^{2+} resulted in partial inhibition (Table 7).

Table 7. Effect of different metal ions on AR-135 xylanase activity.

Metal ion (1 mM)	Relative activity (%)
None	100
NaCl	100
KCl	102
$CaCl_2$	106
$MgSO_4$	93
$BaCl_2$	90
$CoCl_2$	99
$ZnSO_4$	60
$FeCl_3$	43
$HgCl_2$	0
$AlCl_3$	87
$Pb(CH_3)COO^-$	66
$MnCl_2$	94
$CuSO_4$	1

3.2.5. Mode of action. The mode of action of AR-135 xylanase was determined by measuring the rate of viscosity reduction of oat spelt xylan as described above. The enzyme resulted in a rapid reduction in viscosity and corresponding rapid rise in reducing sugar concentration (Fig. 12) indicating that it is an endoxylanase.

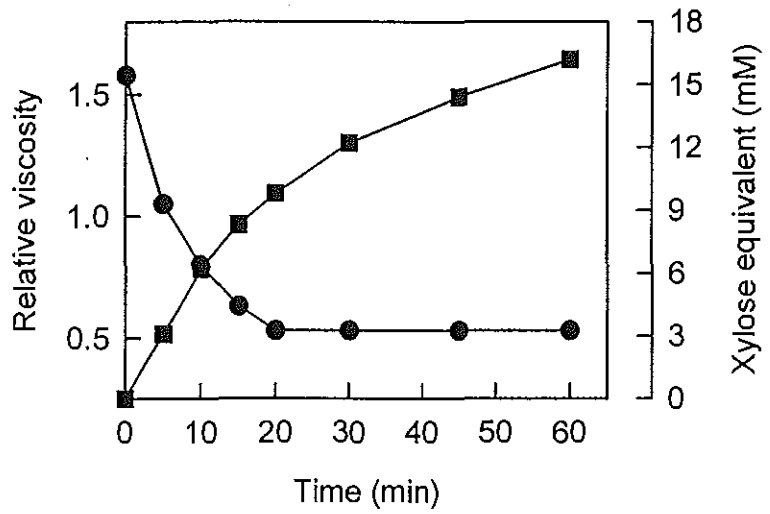


Fig. 12. Viscosity reduction (●) and formation of reducing sugar (■) during degradation of xylan with AR-135 xylanase. The reaction was carried out using 0.5% xylan at 40°C.

3.3. Strain AR-22-1

3.3.1. Purification of the enzyme. The enzyme was purified following ammonium sulfate precipitation, ion exchange and gel filtration chromatography. A summary of the purification procedure is shown in Table 8. The purified enzyme appeared homogenous on SDS-PAGE (Fig. 4c) and its molecular weight was estimated to be 20 kDa.

Table 8. Summary of the purification procedure of AR-22-1 xylanase

Enzyme preparation	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude culture supernatant	1540	2556.0	0.6	1.0	100
Ammonium sulfate	1247	183.4	6.8	11.4	81
DEAE-Sepharose	986	22.2	44.4	74.0	51
Sepadex G-75	354	6.5	54.6	91.0	23

3.3.2. Effect of temperature on activity and stability of the enzyme. The effect of temperatures on activity was determined by assaying the enzyme at different temperature. The optimum temperature for activity was 70°C with 90% of the activity being retained at 75° C (Fig. 13). The effect of temperature on stability was determined by incubating the enzyme at different temperature values using pH 8 or 9 buffer. The enzyme showed good stability at 55°C and 60°C in both pH values (Fig. 14). At 65°C over 68 and 54% of its original was retained after 30 min incubation at pH 8 and 9 respectively.

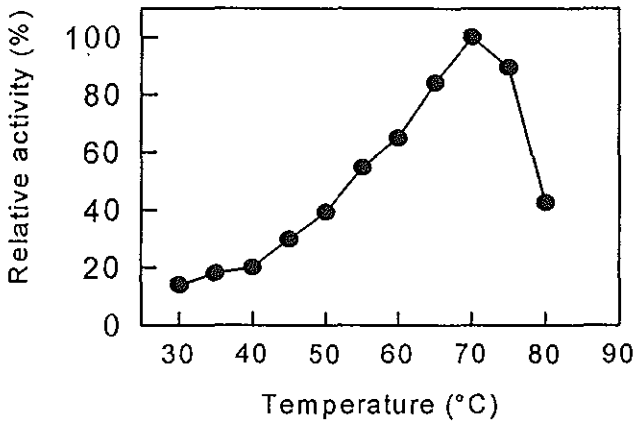


Fig. 13. Temperature profile of AR-22-1 xylanase assayed at different temperature values and pH 8.

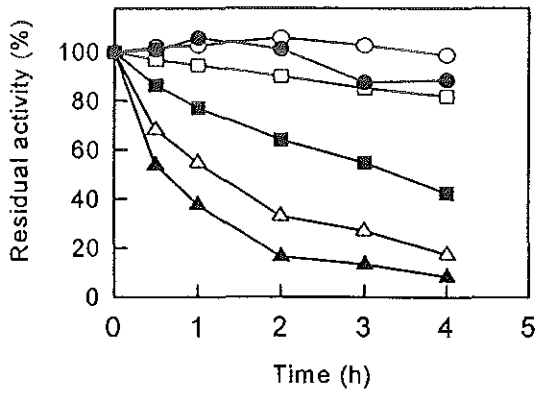


Fig. 14. The effect of temperature on the stability of AR-22-1 xylanase tested at pH 8 (○, ❑ ,Δ) and pH 9 (●,■ ▲) and a temperature of 55°C (○,●), 60°C (❑ , ■), and 65°C (Δ,▲).

3.3.3. Effect of pH on activity and stability. The effect of pH on xylanase activity was measured by assaying the enzyme activity at 50°C in different buffers. The enzyme was active in a broad pH range with an optimum in the range of 8.0 to 9.5 (Fig. 15). Over 75 and 55% of its maximum activity was displayed at pH 10.0 and 10.5, respectively. To study the effect of pH on stability the enzyme was incubated at 50°C for 1 h in different buffers and residual activity was measured following standard assay procedure. Nearly 100% of its original activity was retained in the pH range of 5 to 10 (Fig. 16).

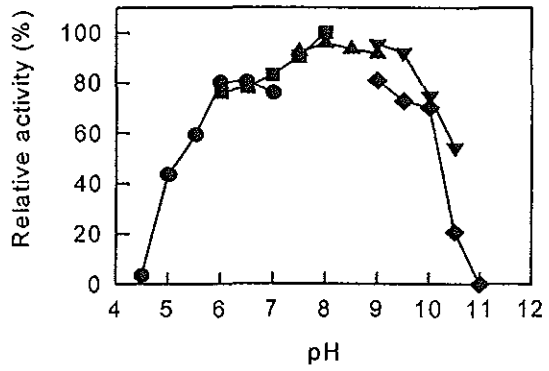


Fig. 15. pH profile of AR-22-1 xylanase. Buffers used were: citrate- phosphate (●); phosphate (■); Tris-HCl (▲); glycine NaOH (▼); and carbonate-bicarbonate (◆).

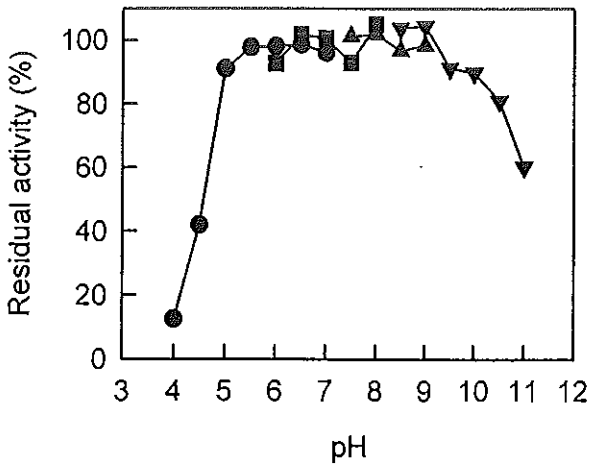


Fig. 16. The effect of pH on the stability of AR-22-1 xylanase. Buffers used were: : citrate-phosphate (◆); phosphate (■); Tris-HCl (▲); glycine NaOH (▼).

3.3.4. Effect of metal ions on activity. The effect of different metal ions on xylanase activity is given in Table 9. The enzyme was inhibited in the presence of Hg^{2+}, Fe^{2+}.

Table 9. Effect of metal ions on xylanase activity, each included at a concentration of 1 mM.

Compound	Residual activity (%)
None	100
NaCl	101
KCl	100
$CaCl_2$	100
$MgCl_2$	100
$CoCl_2$	90
$NiCl_2$	93
$MnCl_2$	53
$ZnSO_4$	90
$CuSO_4$	118
FeSO4	19
$Pb(CH_3COO^-)_2$	35
$HgCl_2$	0
$AlCl_3$	79
$SnCl_2$	102

4. Xylanase production by immobilized cells of *Bacillus* sp. AR-009

4.1. Time course of enzyme production by immobilized cells

Xylanase production by immobilized cells of *Bacillus* sp. AR-009 was followed for a period of 72 h. Enzyme production started after 12 h and reached a maximum after 48 h and remained high up to 72 h (Fig. 17).

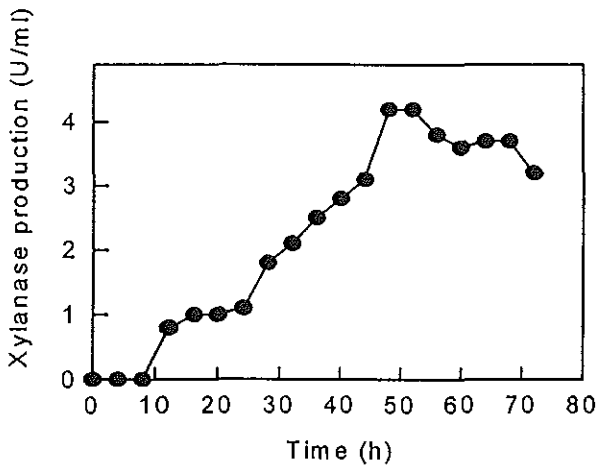


Fig. 17. Time course of xylanase production by agar immobilized cells of *Bacillus* sp. AR-009 cells.

4.2. Xylanase production by repeated batch cultivation of immobilized cells

Xylanase production by immobilized cells was studied for seven consecutive batches. Every 48 h the original culture was removed and the beads washed and resuspended with fresh xylan medium. As shown in Fig. 18 enzyme production was more or less stable in 7 consecutive batches. Through out the whole cultivation cycle the agar beads were stable with out any sign of rupture.

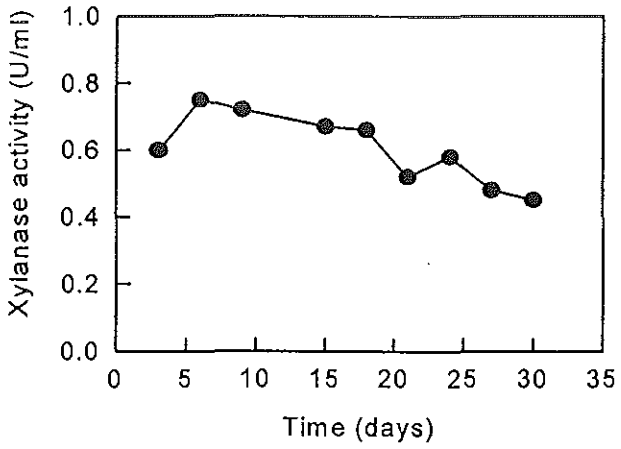


Fig. 19. Xylanase production by immobilized cells of *Bacillus* sp. AR-009 cells using continuous culture.

Table 10. Comparison of xylanase production by free cells and immobilzed cells of *Bacillus* sp. AR-009 under different cultivation conditions.

System	Productivity (U/l/h)	Relative productivity
Free cells, batch	186.4	1.0
Immobilized cells - batch	87.5	0.5
Imm. cells- repeated batch	64.5	0.4
Immob. cells- continuous	800.0	4.3

5. The use of solid state fermentation (SSF) for xylanase production by *Bacillus* sp. AR-009

5.1. Time course of enzyme production

High level of xylanase acivity was produced by *Bacillus* sp. AR-009 grown using wheat bran as a solid support. The time course of enzyme production was followed for a period of 108 h. Maximum production was observed after 72 h (Fig. 20). Further incubation after this time did not show any improvement in the level of enzyme production.

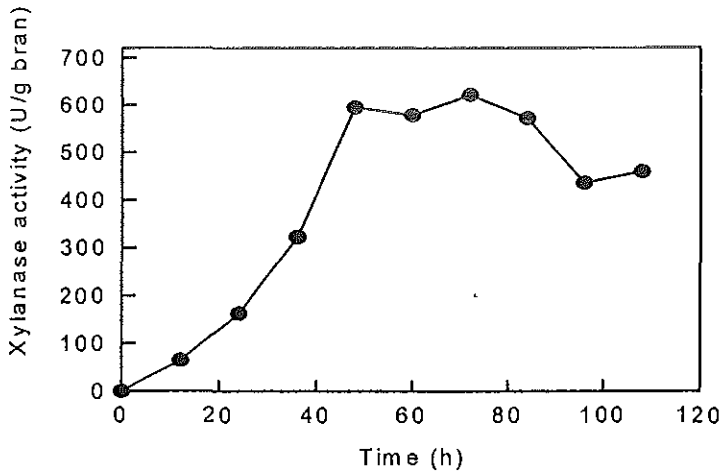


Fig. 20. Time course of xylanase production by *Bacillus* sp. AR-009 grown using solid state fermentation

5.2. Effect of wheat bran to moisture ratio

The highest xylanase production was observed in a wheat bran to moisture ratio of 0.5 to 1.5. With increasing moisture level productivity was decreasing (Fig. 21). At a ratio of 1:3 and above less than 50% of the maximum xylanase production was observed.

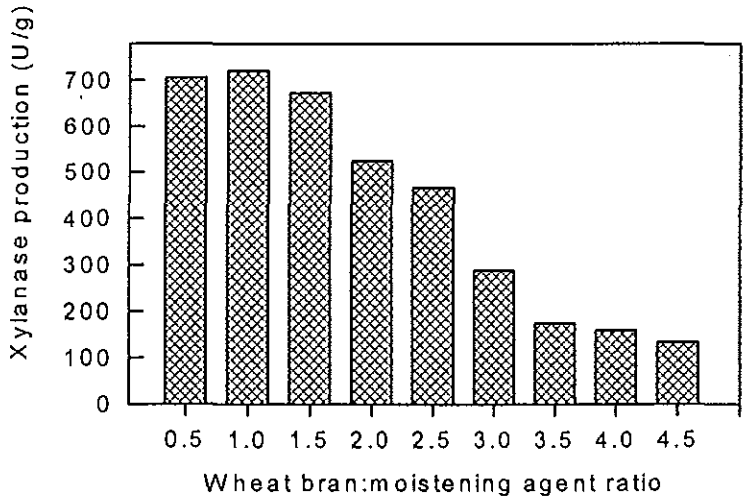


Fig. 21 The effect of wheat bran to moisture ratio on the level of xylanase production by *Bacillus* sp. AR-009.

5.3. Xylanase production at different concentrations of Na_2CO_3

The pH of the medium was adjusted to the alkaline range by adding Na_2CO_3. Maximum xylanase production was obtained at a Na_2CO_3 concentration of 10% (w/w) (Table 11). At 5% (w/w) Na_2CO_3 the organism produced 80% of the productivity at 10%.

Table 11. Effect of three different concentrations of Na_2CO_3 on xylanase production by *Bacillus* sp. AR-009 in solid state fermentation

Na_2CO_3 conc. (% w/w)	Xylanase activity (U/g dry bacterial bran)	Relative production (%)
2.5	496.2	72
5.0	551.2	80
10.0	685.3	100

5.4. Production of other enzymes

In addition to xylanase, *Bacillus* sp. AR-009 grown using SSF produced cellulase and protease activities. However, compared to xylanase activity the level of cellulase and protease activity was very low (Table 12).

Table 12. Production of cellulase and protease in relation to xylanase by *Bacillus* sp. AR-009 in solid state culture.

Enzyme	Enzyme production (U/g dry bacterial bran)
Xylanase	621.3
Cellulase	12.5
Protease	53.0

5.5. Effect of different additives on xylanase production

Xylanase production was repressed upon addition of different sugars. At a concentration of 5% (w/w) xylose and lactose showed significant repression of xylanase production, while the effect of glucose and sucrose was mild (Table 13). The effect of increasing concentration of xylose and glucose on xylanase production was also investigated. At a concentration of 10% (w/w) xylose completely repressed

xylanase production. The effect of glucose was concentration dependent leading to 60% and 90% repression at a concentration of 10% and 15% (w/w), respectively (Fig. 22). Addition of yeast extract, bacteriological peptone, and tryptone to wheat bran showed no significant effect on xylanase production (Table 13).

Table 13. Effect of different additives on xylanase production by *Bacillus* sp. AR-009 in solid state fermentation.

Additive (5% w/w)	Relative xylanase production (%)
None	100
Xylose	23
Glucose	78
Sucrose	74
Lactose	16
Bacteriological peptone	87
Yeast extract	120
Tryptone	105

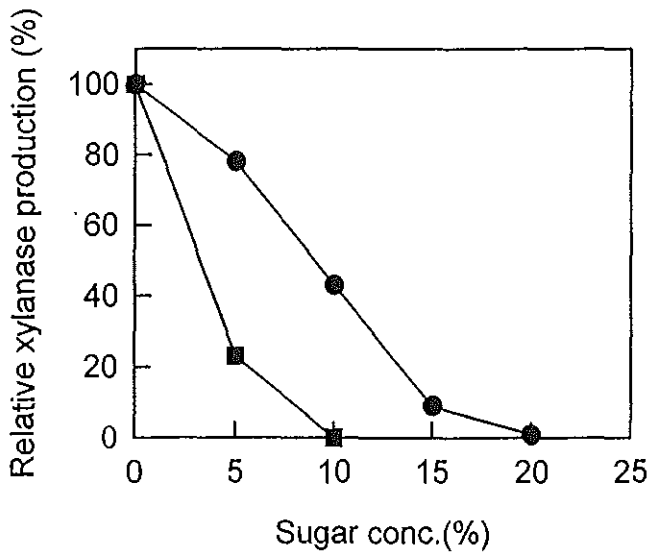


Fig. 22. The effect of increasing concentrations of glucose (●) and xylose (■) on xylanase production by *Bacillus* sp. AR-009 in solid state fermentation.

IV. DISCUSSION

Naturally occurring alkaline habitats, having a stable alkaline pH all the year round offer enormous potential for the isolation of new microbial strains producing novel xylanases. Xylan being too big to enter into the cell, xylanolytic microorganisms adapted to live in such alkaline habitats are expected to produce xylanases that act extracellularly (the enzymes being either cell bound or secreted free in the surrounding medium). Since the pH of the extracellular environment is always alkaline, xylanases from such microorganisms are expected to be operational at alkaline pH values. In recent years alkaline active xylanases attracted considerable attention because of their potential application in different industrial processes (Biely, 1985; Coughlan and Hazlewood, 1993; Nakamura *et al.,* 1993; 1995). To date only few xylanolytic alkaliphiles are known, almost all are isolated from neutral soil samples (Horikoshi and Akiba, 1982; Grant *et al.,* 1990).

In this study xylanase producing alkaliphilic bacterial strains were isolated from Lake Arenguadie, an alkaline soda lake in the Rift Valley of Ethiopia. The lake is very productive with the highest primary productivity ever recorded for a natural habitat (Talling *et al.,* 1973; Wood and Talling, 1988). As a result, a large amount of organic matter is found in the lake. Different groups of heterotrophic bacteria are expected to be present in the lake and be involved in the decomposition of various biological polymers thus playing a vital role in nutrient recycling. Three xylanolytic strains designated as AR-009, AR-135, which were identified as members of the genus *Bacillus* and *Micrococcus,* respectively and a third strain AR-22-1 which is not yet definitively assigned to a specific genus, were isolated from the lake. All the three strains produced alkaline xylanases. The great majority of xylanase producing alkaliphilic strains known so far belong to the genus *Bacillus* (Horikoshi and Akiba, 1982; Grant *et al.,* 1990). *Micrococcus* AR-135, and strain AR-22-1 are among the few exceptions of xylanolytic alkaliphiles that do not belong to the genus *Bacillus.*

Enzyme production by all the three strains was induced in the presence of xylan. In addition, for strains AR-135 and AR-22-1 xylose also served as a good inducer of xylanase production. In the case of AR-135 high level of xylanase activity was detected

in the presence of xylose than in the presence of xylan. No activity was detected when it was grown in the presence of other sugars. Similarly, AR-22-1 produced xylanase activity in the presence of xylan or hydrolysis products of xylan. On the other hand AR-009 produced low level of xylanase activity in all the other sugars tested, the lowest being in xylose containing media. In most xylanolytic microbial strains xylose is known to either suppress enzyme production or support low level of production than xylan (Khanna and Gauri, 1993; Kyu *et al.*, 1994; , Ratto *et al.*, 1992; Yang *et al.*, 1995). In addition, in many strains, including strain AR-009 reported in this study, some basal xylanase activity has been detected in the presence of other carbohydrate sources (Khanna and Gauri, 1993; Kyu *et al.*, 1994; Yang *et al.*, 1995). In this regard the regulatory mechanism(s) of xylanase production by AR-135 and AR-22-1 appear to be different from most other xylanolytic microorganisms.

The time required for maximum enzyme production by all the strains was short, though there was some variation among the three strains. The majority of microbial xylanases studied so far are derived from fungi. Maximum enzyme production by most fungal strains is attained after many hours of cultivation. From the application point of view, faster production of the enzyme could be advantageous which may allow appreciable reduction in the production cost of the enzyme.

Organisms isolated from nature often produce enzymes in low yield. To make the process of enzyme production cost effective high level enzyme producing mutants are derived through mutagenesis (Crueger and Crueger, 1989; Biswas *et al.*, 1990; Smith and Wood, 1991). Another approach is to clone the gene and express it in appropriate host (Page *et al.*, 1996). The level of xylanase production by AR-009 (45 U/ml) was much higher than the level of productivity of any xylanolytic bacteria reported so far. Isolation of high level enzyme producing mutants through mutation and/or optimization of the cultivation conditions for the growth of AR-009 may allow further increase in the level of xylanase production.

Two extracellular alkaline thermostable xylanases, designated as xylA and xylB, were purified from the cell free culture supernatant of *Bacillus* sp. AR-009. The two

enzymes differ in many of their properties. Multiple xylanase production has been reported from a wide variety of microorganisms (Wong, *et al.,* 1988; Biely *et al.,* 1992; Elegir *et al.,* 1994; He, *et al.,* 1994). Biely *et al,* (1992) reported three xylanase isoenzymes from *Streptomyces lividans* which showed considerable difference in their mode of action on different xylan substrates. Given the heterogenous nature of the substrate xylan, the ability to produce multiple forms of xylanases each differing in their specificity and mode of action to ward xylan of different source could be expected to increase the efficiency of the organism in utilizing xylan as a growth substrate under different growth conditions, which under natural conditions is not constant. In addition, the different xylanase isoenzymes are expected to act synergestically thus increasing the efficiency of hydrolysis (Thomson, 1993; He, *et al.,* 1994). Nevertheless, data on multiple xylanase production need to be interpreted with care. The different xylanase isoenzymes could be translation products of separate genes or could appear as a result of proteolytic degradation of one enzyme during purification.

Xylanases produced by most alkaliphiles reported to date have optimum pH around neutrality (Okazaki *et al.,* 1984; Tsujibo *et al.,* 1990; Dey *et al.,* 1992; Park *et al.,* 1992). Nakamura *et al.* (1993) reported the first alkaline xylanase produced by *Bacillus* sp. strain 41M-1 which had optimum temperature and pH of 50°C and 9, respectively. The thermal stability of this enzyme was reported to be low losing about 90% of its activity after 30 min heating at 60°C, pH 9.

The only alkaline active thermostable xylanase reported so far is that produced by *Bacillus* sp. TAR-1 having a temperature optimum of 70°C at pH 9 and 75°C at pH 7. The optimum pHs of xylanases reported to date drop with increasing temperature. In this regard xylB of *Bacillus* sp. AR-009 is probably unique in having an alkaline pH optimum with increasing temperature. These are desirable properties for application in different industrial processes. Further study of xylB might give information on the molecular basis of stability and activity of xylanases at alkaline pH and elevated temperature.

Based on the amount of the two enzymes recovered at the final stage of the purification step, xylB accounts for less than 3% of the total xylanase activity. This could be a a reflection of the level expression of the two enzymes by the organism or might also be a result of the purification procedure. Considering the potential usefulness of xylB for different applications, understanding the regulatory mechanisms for the secretion of the two enzymes and optimization of the cultivation conditions may allow to increase its level of production. Another approach could be to clone the gene coding for xylB and express it in an appropriate host. Cloning of the gene may offer an additional advantage of improving some properties of the enzyme through site-directed or random mutagenesis.

The enzyme from AR-22-1 was also a thermostable alkaline xylanase optimally active in a broad pH range with optimum activity at pH of 8-9 and a temperature of 70°C. Its activity and stability at alkaline pH and elevated temperature makes it ideal for a variety of applications. It is possible to further improve the properties of the enzyme and/or the level of production using genetic manipulation.

Xylanase AR-135 was active in a broad pH range with an optimum at pH 7.5 - 9.0 (Fig. 2A) and retained up to 60% of the maximum activity at pH 11. On the other hand the optimum temperature for activity and stability was low. Though activity and stability of AR-135 xylanase at alkaline pH is a desirable property for many applications; its low thermal stability could be a disadvantage for some applications, such as in the pulp and paper industries where alkaline xylanases having good activity and stability at elevated temperature are needed (Zamost *et al.*, 1991). One possibility to improve thermal stability could be to carry out site directed or random mutagenesis of the cloned gene. Previously such an approach has been shown to give an appreciable increase in the thermal stability of xylanase A of *Streptomyces lividans* 1326 (Moreau, *et al.*, 1994).

The four xylanases derived from the three strains differ in their response to inhibition by metal ions. XylA and xylB of *Bacillus* sp. AR-009 and AR-22-1 xylanase were

partially inhibited in the presence of Mn^{2+} while no inhibition was observed for AR-135 xylanase. On the other hand, in the presence of Cu^{2+} the activity of AR-135 xylanase was completely inhibited while the activity of the other three enzymes was not affected. Resistance to inhibition by different metal ions differ among xylanases derived from different organisms and even isoenzymes of the same organism (Tsujibo *et al.,* 1990; Park *et al.,* 1992; Moreau *et al.,* 1994; Winterhalter and Lieb, 1995). For example, xylanase R of *Bacillus* sp. TAR-1 was reported not be affected by Fe^{2+}, Fe^{3+} while Cu^{2+} resulted in a 60% loss of activity (Nakamura *et al.,* 1995). Cu^{2+} has also been shown to inhibit about 60% of *Thermomonospora alba* ULJB1 xylanase (Blanco *et al.,* 1997), and over 90% of *Bacillus amyloliquefaciens* xylanase (Breccia *et al.,* 1998). Although Fe^{2+} and Fe^{3+} at a concentration of 1 mM have been shown to partially inhibit the activities of many xylanases, no complete inhibition was reported previously. The complete inhibition of activity of xylA and xylB of *Bacillus* sp. AR-009 by these ions seems to be unique.

In recent years xylanases find important application in the pulp and paper industry. The majority of xylanases reported so far showed optimum activity in the acidic or neutral pH range (Coughlan *et al.,* 1993). From the application point of view xylanases active and stable in the alkaline range are very important.

In the Kraft process of pulp production, the pulp prior to the normal bleaching operation has an alkaline pH and high temperature (Zamost *et al.,* 1991; Viikari *et al.,* 1994). The use of most commonly known xylanases for this application necessitates lowering the temperature to around 50°C and adjusting the pH to acidic or neutral values. For subsequent bleaching operations the temperature needs to be raised to around 70-75°C and the pH readjusted to the alkaline range (Wong and Saddler, 1993). This process of cooling and reaheating, and lowering and raising the pH of the large pulp mass is very expensive, taking considerable time, energy and chemicals. As a result enzyme based pulp bleaching is not attractive for many pulp and paper mills.

To overcome this problem in recent years attention has been focused on the isolation of xylanase producing extremophiles from different extreme habitats. As a result up to

now a number of thermostable xylanase producing thermophilic organisms have been isolated in different laboratories (Dahlberg, 1996; Sunna and Anthranikian, 1996). However, the pH optima of all thermostable xylanases of thermophiles known so far are in the acidic or neutral range. Another approach was isolation of xylanolytic alkaliphilic and alkalitolerant microorganisms that could produce alkaline active xylanases. Nevertheless, the majority of alkaliphiles and alkalitolerant microorganisms produce xylanases optimally active around neutrality and a temperature of 50°C or below (Okazaki *et al.,* 1985; Blanco *et al.,* 1995; Dey *et al.,* 1992; Kang *et al.,* 1996 ; Nakamura *et al.,* 1993; Park *et al.,* 1992; Ratto *et al.,* 1992). Only few alkaliphilic bacterial strains are known to produce xylanases with optimum activity at pH values greater than 8 (Nakamurae *et al.,* 1995). In enzyme-assisted bleaching of kraft pulp an enzyme active and stable at alkaline pH and elevated temperature is very important. The use of such enzymes is expected to greatly reduce the need for pH and temperature readjustments of the large pulp mass thus allowing substantial reduction in the cost of enzyme assisted pulp bleaching operations. In this regard xylanases produced by AR-009 and AR-22-1 have tremendous potential for pulp bleaching application. XylB of AR-009 is especially attractive in that up to 75°C its optimum was increasing with increasing pH.

Another area of application for alkaline xylanases is in the hydrolysis of hemicelluosic biomass to simple sugars (Hespell, *et al.,* 1997; Wong and Saddler, 1993). World wide several million tons of xylan is released annually in the form of agricultural, industrial, and municipal waste. Because xylan is soluble at alkaline pH, the high activity and stability of the four xylanases at alkaline pH and relatively high temperature will be of great advantage for the hydrolysis of such wastes.

In the process of pulp production using the kraft process a large amount of xylan containing waste is released in the effluent. Because the effluent has an alkaline pH, the use of alkaline xylanases for the hydrolysis of such waste is expected to allow development of a cost effective saccharification processes by reducing the amount of acid required for pH readjustment and thus lowering the cost of ion exchange media needed during down stream processing.

In the past the study of lignocellulosic bioconversion was actively pursued mainly in the developed nations. However, at present less attention is given to the study of lignocellulosic bioconversion. One reason may be the complexity of the lignocellulosic substrate discouraging further development. Another reason could be that in developed nations starch obtained from cereal sources, is readily available with cheap price which may thus discourage development of lignocellulosic based methods. However, with growing awareness of environmental pollution and the increasing costs incurred in treating organic pollutants, the increasing demand for cereals as food for the ever growing world population, and progress in the understanding of biological processes, in the future lignocellulosic bioconversion may provide a better alternative. Under such conditions xylan degrading enzymes, together with other plant cell wall polymer degrading enzymes, could play a vital role to bring about complete hydrolysis of plant biomass. Considering the alkaline solubility of xylan and the alkaline nature of some hemicellulosic wastes xylanases from the 3 strains discussed above have tremendous potential.

After a given organism producing enzymes of potential importance is isolated from nature, the next challenge is how to produce the enzyme in a cost effective way. In the production of commercial enzymes, currently the growth substrate account 30-40% of the production cost (Hinman, 1994). Separation of the cell mass from the culture fluid, concentrating the enzyme, which is often found in low concentration, through downstream processing is known to account a huge part of the over all production cost (Mattiasson, 1986; Hahn-Hagerdal, 1986). Therefore, finding the best way of cultivation reducing expenses in one or more of the above processes is expected to have a significant effect on the over all production cost.

Unlike most organisms isolated from nature, AR-009 produced high level of xylanase activity. For this reason, this strain was selected to study the effect of two cultivation conditions (*viz.* immobilization and solid state fermentation) for the production of xylanase.

Given the potential usefulness of the xylanases produced by *Bacillus* sp. AR-009, development and optimization of methods for the production of alkaline active thermostable xylanases by immobilized cells could have significant economic and technical advantages. In this study *Bacillus* sp. AR-009 cells were immobilized using agar as the immobilization matrices. Immobilized cells produced appreciable level of xylanase activity in batch and continuous culture.

In a batch culture of immobilized cells, xylanase activity was detected after 12 h and reached to a maximum after 48 h. Using free cells measurable xylanase activity was detected starting from 4 h. One possibility for the delay in xylanase production by immobilized cells may be a result of retarded growth of cells up on immobilization. A similar observation was also reported for other organisms (Kanasawud, *et al.*, 1989; Singh *et al.*, 1990; Klimberg, *et al.*, 1990; Mamo and Gessesse, 1997). Another difference observed was that the level of enzyme production was lower in the case of immobilized cells than free cells. One explanation may be diffusional limitation of oxygen access to cells at the interior of the beads. Diffusional limitation of oxygen is known to affect growth and productivity of immobilized cells (Mattiasson, 1983a,b; Omar, 1993). In the case of immobilized *Bacillus* sp. AR-009 cells the low solubility of xylan may further complicate the situation. Because xylan can not easily diffuse in to the interior of the agar beads, only cells exposed at the surface of the beads may probably be induced and produce the enzyme. Cells deep in the interior of the agar bead may remain idle being nourished by the other soluble medium components. In the future a search for low molecular weight soluble substrates that can induce xylanase production by *Bacillus* sp. AR-009 cells may help to improve the level of xylanase production by immobilized cells. One possibility, for example, may be the use of xylooligosacchrides after partial or complete enzymatic saccharification.

The agar beads showed remarkable stability for an extended period of time. Xylanase production was more or less constant in 7 cycles of repeated batch cultivation. This indicates the possibility of using agar immobilized cells of *Bacillus* sp. AR-009 for repeated xylanase production. Immobilized *Bacillus* sp. AR-009 cells were also used for continuous production of xylanase for a period of one month with out any decline

in productivity. One advantage of using immobilized cells for extracellular enzyme production is the possibility of using it for continuous or repeated enzyme production for extended period of time which will have significant economic advantage. Under such conditions the risk of contamination will be lower, and the cost of down stream processing will be reduced. Further understanding of the physiology of immobilized *Bacillus* sp. AR-009 cells and improvement in medium composition for better enzyme induction may lead to even higher level of xylanase production.

Another method of enzyme production studied was solid state fermentation. *Bacillus* sp. AR-009 produced high titre of xylanase activity when grown using SSF with wheat bran serving as a solid substrate. Maximum enzyme production was obtained at a Na_2CO_3 concentration of 10% (w/w) and at a lower wheat bran to moisture ratio (between 1:0.5 to 1:1.5). Other workers also showed high level of enzyme production at lower solid substrate to moisture ratio by different bacterial strains grown using SSF (Lonsane and Ramesh, 1990; Ramesh and Lonsane, 1987; Tobey *et al.*, 1976). Increasing moisture level is believed to reduce the porosity of the wheat bran thus limiting oxygen transfer (Lonsane and Ramesh, 1990). The fact that the organism grows and produces maximum xylanase activity at lower wheat bran to moisture ratio and at an alkaline pH will offer significant advantage in reducing the risk of contamination. Most bacterial species are unable to grow at reduced moisture level and alkaline pH. On the other hand, though many fungi are capable of growing on moist substrates, their optimum growth pH is usually below neutrality (mostly pH 4 to 6).

At the optimum growth condition for SSF high level of xylanase production was obtained. High titre amylase (Ramesh and Lonsane, 1989) and xylanase (Archana and Satyanarayana, 1997) production was also reported for other *Bacillus* strains grown using SSF. The enzyme from the SSF culture can be extracted with two to three volumes of liquid thus giving a highly concentrated enzyme. The volume of liquid used for extraction can be further reduced by employing more efficient extraction procedures (Ramakarishna *et al.*, 1982). To get the equivalent amount of enzyme using SMF a large volume of fermentation liquid need to be handled. Thus, cell separation from the fermentation liquid using centrifugation or microfiltration and

subsequent removal of water for enzyme concentration account for a large proportion of the production cost in SMF (Datar, 1986; Mattiasson, 1986). In addition, the large volume of liquid waste that must be disposed off after appropriate waste treatment operations further increases the production cost (Lonsane and Ramesh, 1990; Hahn-Hagerdal, 1986). Therefore, the low volume of liquid to be handled during the production of *Bacillus* sp. AR-009 xylanase using SSF means a significant reduction in down stream processing and subsequent waste treatment costs.

Xylanase production by solid state fermentation may have a very interesting potential application in the animal feed industry. The solid substrate could be easily dried and mixed with other feed components. In this way the cost of enzyme production will be substantially reduced.

In addition to xylanase, the organism produces low level of cellulase and protease activities. The presence of a protease may affect the stability of the xylanase during extraction and storage. The use of appropriate protease inhibitors during extraction or selection of protease deficient mutants may help to solve the effect of protease. The presence of cellulase activity together with xylanase has been considered a disadvantage in enzyme assisted pulp bleaching because of its adverse effect on pulp strength. However, some studies showed that treatment of pulp with cellulases, provided care is taken to avoid excessive degradation, has a beneficial effect by improving pulp freeness and other properties (Kamaya, 1996). It has also been shown that different cellulases differ in their effect on the pulp (Pere *et al.,* 1995). Therefore, in view of potential application for enzyme assisted pulp bleaching, the beneficial or harmful effects of *Bacillus* sp. AR-009 cellulase on the pulp remains to be evaluated.

Addition of different sugars to wheat bran resulted in mild to strong repression of xylanase production in SSF culture. Xylose strongly repressed xylanase production at lower concentration while the effect of glucose was found to be concentration dependent, being mild at lower concentration (5% w/w) and become much more pronounced at a concentration of 15% (w/w) and above. Addition of organic nitrogen sources showed no significant effect on productivity. This shows that wheat bran has

enough nutrients to support high titre xylanase production by *Bacillus* sp. AR-009. Many studies also showed that wheat bran contains a good balance of nutrients to support growth of many microorganisms (Solis-Pereira *et al.*, 1993; Babu and Satyanarayana, 1995). The fact that no addition of expensive media is required will have important economic advantage.

V. CONCLUDING REMARKS

Alkaline active xylanases have enormous potential for application in different industrial processes. Currently there are two possible ways of obtaining enzymes having the required property: 1) improvement of existing enzymes through protein engineering ; or 2) screening of new microbial strains from nature which produce enzymes with the required property for a given application condition. At present the latter is considered to be the best alternative. This is especially important when one considers the fact that up to now less than 1% of the microorganisms estimated to exist on earth is properly studied.

Alkaliphilic microorganisms are considered as the best sources of alkaline active enzymes. However, almost all alkaliphiles known so far were isolated from neutral soil and xylanases produced by such organisms are optimally active below the neutral pH range. Xylanases from the three strains isolated in this study were optimally active in the alkaline pH range. In addition the enzymes produced by *Bacillus* sp. AR-009 and strain AR-22-1, were optimally active at elevated temperature, properties considered very important for a variety of applications. This shows the great potential of alkaline habitats of the Great African Rift Valley for the isolation of novel microbial strains producing enzymes of tremendous potential for biotechnological applications. In spite of their potential, however, up to now extremely little effort has been made to know the diversity of alkaliphilic microorganisms from naturally occurring alkaline habitats. In addition to this the situation may be further complicated by the fact that up to now out of the total microbial population estimated to exist in every environment only a very small fraction can be cultured using commercially available media. As a result knowing the actual diversity of microorganisms in the alkaline soda lakes of the Great African Rift Valley may not be easy. A new development in the last couple of years is

the ability to isolate and clone DNA fragments from nature directly and screen these clones for the enzyme of interest. Using this method alkaline soda lakes are expected to give novel xylanases and other enzymes of potential biotechnological importance.

After a given organism producing enzymes of potential biotechnological importance is obtained, the next step is the development of cost effective method for production of the enzyme. In this study an attempt was made to use immobilization and solid state fermentation for the production of xylanase by *Bacillus* sp. AR-009. Although agar immobilized cells were used for the continuous production of xylanase for extended period of time, the system requires further optimization. On the other hand high level of xylanase production was obtained when the organism was grown using SSF.

Xylanase production by alkaliphlic *Bacillus* sp. AR-009 using solid state fermentation will have several advantages. High level enzyme production at a reduced moisture level and alkaline pH could minimise the risk of contamination. The enzyme can be extracted in a minimum volume of extraction liquid thus decreasing the total volume of liquid to be handled during product upgrading and subsequent disposal of the waste after appropriate waste treatment operations. Wheat bran supplied enough nutrients with out any need for addition of expensive supplements. All these combined together could greatly reduce the over all cost of xylanase production.

VI. REFERENCES

Antoniou TC and Marquardt RR (1981). Influence of rye pentostans on the growth of chicks. *Poult. Sci.* **60**:1898-1904.

Antoniou TC and Marquardt RR (1982). Utilization of rye diets by chicks as affected by lipid type and level of penicillin supplementation. *Poult. Sci.* **61**: 107-116.

Aono R and Ohtani M (1990). Loss of alkalophily in cell-wall-component-defective mutants derived from alkaliphilic Bacillus sp. C-125. *Biochem. J.* **266**: 933-936.

Aono R, Ito M, and Horikoshi K (1993). Occurrence of teichuronopeptide in cell walls of group 2 alkaliphilic *Bacillus* sp. *J. Gen. Microbiol.* **139**:2739-2744.

Aono R, Ito M, Joblin KN and Horikoshi K (1995). A high cell wall negative charge is necessary for the growth of the alkaliphilic *Bacillus lentus* C-125 at elevated pH. *Microbiology* **141**: 2955-2964.

Archana A and Satyanarayana T (1997). Xylanase production by thermophilic *Bacillus lichenifomis* A99 in solid state fermentation. *Enzyme Microb. Technol.* **21**, 12-17.

Babu K R and Satyanarayana T (1995). α-Amylase production by thermophilic *Bacillus coagulance* in solid state fermentation. *Proc. Biochem.* **30**, 305-309.

Bajpai P and Bajpai PK (1992). Biobleaching of kraft pulp. *Proc. Biochem.* **27**: 319-325.

Bastawde KB. (1992). Xylan structure, microbial xylanases, and their mode of action. *World J. Microbiol. Biotechnol.* **8**: 353-368.

Bedford MR (1996). The effect of enzymes on digestion. *J. Appl. Poult. Sci.* **5**: 370-378.

Bedford MR and Morgan AJ (1996). The use of enzymes in poultry diets. *World Poul.Sci. J.* **52**: 61-68.

Belay A, Kato T, and Ota, Y. (1996). *Spirulina (Arthrospira)*: potential application as an animal feed supplement. *J. Appl. Phycol.* **8**: 303-311.

Belay A, Ota Y, Miyakawa K, and Shimamatsu H (1993). Current knowledge on the potential health benefits of *Spirulina*. *J. Appl. Phycol.* **5**: 235-241.

Biehl RR and Baker DH (1997). Microbial phytase improves amino acid utilization in young chicks fed diets based on soyabean meal but not diets based on peanut meal. *Poult. Sci.* **76**: 355-360.

Biely P (1985). Microbial xylanolytic systems. *Trends Biotecnol.* **3**: 286-290.

Biely P, Kluepfel D, Morosoli R and Shareck F (1992). Mode of action of three β-1,4-xylanases of *Streptomyces lividans*. *Biochim. Biophys. Acta* **1162**: 246-254.

Biswas, SR, Jana SC, Mishra AK, & Nada G (1990). Production , purification, and characterization of xylanase from a hyperxylanolytic mutant of Aspergillus ochraceus. *Biotechnol. Bioeng.* **35**: 244-251.

Blanco J, Coque JJR, Velaso J and Martin JF (1997). Cloning, expression in *Streptomyces lividans* and biochemical characterization of a themostable endo-β-1,4-xylanase of *Thermomonospora alba* ULJB1 with cellulose-binding ability. *Appl. Microbiol.* 48: 208-217.

Blanco A, Vidal T, Colom JF and Pastor FIJ (1995). Purification and properties of a xylanase A from alkali-tolerant *Bacillus* sp. strain BP-23. *Appl. Env. Microbiol.* **61**: 4468-4470.

Booth IR (1985). Regulation of cytoplasmic pH in bacteria. *Microbiol. Rev.* **49**: 359-378.

Breccia JD, Sineriz F, Baigori MD, Castro GR, Hatti-Kaul R (1998). Purification and characterization of a thermostable xylanase from *Bacillus amyloliquefaciens. Enz. Microb. Technol.* **22**: 42-49.

Broda P (1992). Biotechnology in the degradation and utilization of lignocellulose. *Biodegradation* **3**: 219-238.

Brodelius P and Vandamme E J (1987). Immobilized cell systems. In: *Biotechnology*. (Rhem HJ and Reed G. eds). Vol. 7a, pp: 407-463, Weinheim: VCH Verlagsgesellschaft.

Campbell GL and Bedford MR (1992). Enzyme applications for monogastric feeds: a review. *Can. J. Anim. Sci.* **72**: 449-466.

Cheng J, Guffanti AA, and Krulwich TA (1994). The chromosomal tetracycline resistance locus of *Bacillus subtilis* encodes a Na^+/H^+ antiporter that is physiologically important at elevated growth pH. *J. Biol. Chem.* **269**: 27365-27371.

Christophersen C, Andersen E, Jakobsen TS and Wagner P (1997). Xylanases in wheat separation. *Starch/Starke* **49**: 5-12.

Christov LP and Prior BA (1993). Xylan removal from dissolving pulp using enzymes of *Aureobasidium pullulans. Biotechnol. Lett.* **15**: 1269-1274.

Collins MD, Lund BM, Farrow JAE, and Schleifer KH (1993). Chemotaxonomic study of an alkaliphilic bacterium, *Exiguobacterium auranitacum* gen. nov. sp. nov. *J. Gen. Microbiol.* **129**: 2037-2042.

Colwell RR (1997). Microbial diversity: the importance of exploration and concervation. *J. Ind. Microbiol. Biotechnol.* **18**: 302-307.

Coughlan MP, and Hazlewood, GP (1993). β-1-4-D-Xylan-degrading enzyme systems: bochemistry, molecular biology and applications. *Biotechnol. Appl. Biochem.* **17**: 259-289.

Coughlan MP, Tuohy MG, Filho XF, Puls J, Claessens M, Vrsanska m and Hughes MM (1993). Enzymological aspects of hemicellulases with emphasis on fungal systems. In: *Hemicellulose and Hemicellulases*. (Coughlan MP and Hazlewood GP, eds.) Portland Press, London. pp. 55-84.

Crueger W and Crueger A (1989). *Biotechnology: a text book of industrial microbiology*. 2nd ed, Sinauer Associates, Inc., Sundurland.

Dahlberg, L (1996). Thermostable xylanases from *Rhodothermus marinus*. *PhD Thesis*, Lund University, Sweden.

Daneault C, Leduc C, and Valade JL (1994). The use of xylanases in kraft pulp bleaching. A review. *Tappi J.* **77**: 125-131.

Datar R. (1986). Economics of primary separation steps in relation to fermentation and genetic engineering. *Proc. Biochem.* **21**: 19-26.

Dey D, Hinge J, Shendye A and Rao M (1992). Purification and properties of extracellular endoxylanases from alkaliphilic thermophilic *Bacillus* sp. *Can. J. Microbiol.* **38**: 436-442.

Dobreva E, Ivanova V, Tonkova A, Radulova E (1996). Influence of the immobilization conditions on the efficiency of *alpha*-amylase production by *Bacillus licheniformis*. *Proc. Biochem.* **31**: 229-234.

Duckworth AW, Grant WD, Jones BE, & van Steenbergen R (1996). Phylogenetic diversity of soda lake alkaliphiles. *FEMS Microbiol. Ecol.* **19**: 181-191.

Elegir G, Szakacs G, and Jeffries TW (1994). Purification, characterisation, and substrate specificities of multiple xylanases from *Streptomyces* sp. strain B-12-2. *Appl. Env. Microbiol.* **60**: 2609-2615.

Federici RG, Federici F, and Peruccioli M (1990). Continous production of glucoamylase by immobilized cells of *Aurobacidium pullulans*. *Biotechnol.Lett.* 12: 661-666.

Felix R and Vllettaz J-C (1983). In: *Industrial enzymology: the application of enzymes in industry*. (Godfrey T and Reichelt JR, eds.) Macmillan Publ. Ltd. Basingstoke, pp. 410-421.

Florenzano G, Sili C, Pelosi E, and Vincenzini M (1985). *Cynospira rippkae* and *Cyanspira capsulatus* (gen. novo. sp. novo.) a new filamentous heterocystous cyanobacterium from lake Magadi (Kenya). *Arch. Microbiol.* **140**: 301-307.

Gee JM, Lund BM, Metcalf G and Peel JL. (1980). Properties of a new group of alkaliphilic bacteria. *J. Gen. Microbiol.* **117**: 9-17.

Gessesse A (1998). Novel enzymes of potential biotechnological importance fom extremophiles of East African Rift Valley. Paper presented at the Regional Workshop in Biotechnology, September 16-18, Nairobi, Kenya.

Gessesse A and Gashe BA (1997). Production of alkaline protease by an alkaliphilic bacteria isolated from alkaline soda lake. *Biotechnology Letters* **19**: 479-481.

Godfrey T (1983). In: *Industrial enzymology: the application of enzymes in industry*. (Godfrey T and Reichelt JR, eds.) Macmillan Publ. Ltd. Basingstoke, pp. 424-427.

Grant WD (1989a). *Natronobacterium*. In: *Bergey's Manual of Systematic bacteriology*. Vol. 3. Staley JT, Bryant, MP, Pfennig N, Holt JG eds.). Williama and Williams. pp. 2231-2232.

Grant WD (1989b). *Natronococcus*. In: *Bergey's Manual of Systematic bacteriology*. Vol. 3. Staley JT, Bryant, MP, Pfennig N, Holt JG eds.). Williama and Williams. pp. 2232-2233.

Grant WD (1992). Alkaline environments. In: *Encyclopedia of Microbiolgy*, Vol 1. Academic Press, New York, pp. 73-80.

Grant WD, Horikoshi K (1992). Alkaliphiles: ecology and biotechnological applications. In: *Molecular biology and biotechnology of exteremophiles*. Herbert RA and Sharp RJ eds. Chapman and Hall, New York, pp. 141-162.

Grant, WD and Horikoshi K (1992). Alkaliphiles: ecology and biotechnological applications. In: Herbert, R. A. and Sharp, R. J. eds. *Molecular biology and biotechnology of extremophiles*. Chapman and Hall, New York, pp. 243-262.

Grant WD, Mwatha WE, and Jones BE (1990). Alkaliphiles: ecology, diversity and applications. *FEMS Microbiol. Rev.* **75**: 255-270.

Groth I, Schumann P, Rainey FA, Martin K, Schuetze B and Augsten K. (1997). *Bogoriella caseilytica* gen. novo. sp. nov., a new alkaliphilic actinomycete from soda lake in Africa. *Int. J. Syst. Bacteriol.* **47**: 788-794.

Guffanti AA and Kulwich TA (1994). Oxidative phosphorelation by ADP+Pi- loaded vesicles from alkaliphilic *Bacillus firmus* OF4. *J Biol. Chem.* **269:**21576-21582.

Hamamoto T, Hashimoto M, Hino M, Kitada M, Seto Y, Kudo T and Horikoshi K (1994). Characterization of a gene responsible for the Na^+/H^+ antiporter system of alkaliphilic B*acillus* sp. strain C-125. *Mol. Microbiol.* **14**: 939-946.

Hahn-Hagerdal, B. (1986). Water activity: a possible external regulator in biotechnological processes. *Enzyme Microb. Technol.* **8**: 322-327.

He L, Bickerstaff GF, Paterson A, and Buswell JA (1994). Evaluation of catalytic activity and synergism between two xylanase isoenzymes in enzymatic hydrolysis of two separate xylans in different states of solubility. *Enzyme Microb. Technol.* **16**: 696-702.

Hecky RE and Kilham P (1973). Diatomes in alkaline, saline lakes: ecology and geochemical implications. *Limnol. Oceanog.* **18**: 53-71.

Herbers K, Wike I and Sonnewald U (1995). A thermostable xylanase from *Clostridium thermocellum* expressed at high levels in the apoplast of transgenic tobacco has no detrimental effects and is easily purified. *Biotechnology* **13**: 63-66.

Hespell RB, O'Bryan PJ, Moniruzzaman M, and Bothast RJ (1997). Hydrolysis by commercial enzyme mixtures of AFEX-treated corn fiber and isolated xylans. *Appl. Biochem. Biotechnol.* **62**: 87-97.

Hicks DB and Krulwich TA (1990). Purification and reconstitution of the F_1F_o-ATP synthase from alkaliphilic *Bacillus firmus* OF4. Evidence that the enzyme translocates H^+ but not Na^+. *J. Biol. Chem.* **265**: 20547-20554.

Hicks DB and Krulwich TA (1995). The respiratory chain of alkaliphilic bacteria. *Biochim. Biophys. Acta* **1229**: 303-314.

Hinman, R. L. (1994). The changing face of the fermentation industry. *Chemitech*. June, 45-48.

Hofmann A and Dimroth P. (1990). *Eur. J. Biochem.* **194**:423-430.

Hofmann A and Dimroth P. (1991). The ATPase of *Bacillus alcalophilus*: reconstitution of energy transducing functions. *Eur. J. Biochem.* **196**: 493-497.

Hofmann A, Laubinger W and Dimroth P. (1990). *Biochim. Biophys. Acta* **1018**: 206-210.

Horikoshi K and Akiba T (1982). *Alkalophilic microorganisms*, Springer, Berlin.

Horikoshi, K (1996). Alkaliphiles- from an industrial point of view. *FEMS Microbiol. Rev.* **18**: 259-270.

Hugenholtz P and Pace NR (1996). Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends Biotechnol.* **14**: 190-197.

Ito M, Guffanti AA, Zemsky J, Ivey DM, Krulwich TA (1997). Role of the nhaC-encoded Na^+/H^+ antiporter of alkaliphilic *Bacillus firmus* OF4. *J. Bacteriol.* **179**: 3851-3857.

Jackisch B and Jeroch, H (1990). Studies for the improvement of the feed value of fattening feed for broilers rich in rye by enzyme supplementats. *Arch. Anim. Nutr.* **40**: 11091118.

Janecek S (1993). Strategies for obtaining stable enzymes. *Proc. Biochem.* **28**: 435-445.

Jeffries TW (1990). Biodegradation of lignin-carbohydrate complexes. *Biodegradation* **1**: 163-176.

Jones BE, Grant WD, Collines NC, and Mwatha WE. (1994). Alkaliphiles: diversity and identification. In: *Bacterial diversity and systematics.* Priest FG, Ramos-Cormenzana A and Tindall BJ eds. Plenum Press, New York. pp. 195-230.

Kiy T and Tiedtke A (1993). Effects of immobilization on growth, morphology, and DNA content of the ciliated protozoan *Tetrahymena thermophila*. *FEMS Microbiol. Lett.* **106**: 117-122.

Klingberg M, Vorlop KD, and Antranikian G (1990). Immobilization of anaerobic thermophilic bacteria for the production of cell free thermostable α-amylase and pullulanases. *Appl. Microbiol. Biotechnol.* **33**: 494-500.

Kringstad, KP (1989). Environmental aspects on the future developments of pulp bleaching. In: *Wood processing and utilization*. (Kennedy JF, Phillips GO and Williams PA, eds), John Wiley and Sons, New York. pp. 31-62.

Krulwich TA and Guffanti AA (1992). Proton coupled process in extremely alkaliphilic bacteria. *J. Bioenerg. Biomemb.* **24**: 587-599.

Krulwich TA (1995). Alkaliphiles: 'basic' molecular problems of pH tolerance and bioenergetics. *Mol. Microbiol.* **15**: 403-410.

Krulwich TA, Ito M, Gilmour R and Guffanti AA (1997). Mechanisms of cytoplasmic pH regulation in alkaliphilic strains of Bacillus. *Extremophiles* **1**:163-169.

Kuchner O and Arnod FH (1997). Directed evolution of enzyme catalysts. *Trend Biotechnol.* **15**: 523-530.

Kyu, KL, K Ratankhanokchai, D Uttapap, and M Tanticharoen. 1994. Induction of xylanase in *Bacillus circulans* B_6. *Bioresource Technol.* **48**: 163-167.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**: 680-685.

Langworthy TA (1978). Microbial life in extereme pH values. In: *Microbial life in extereme environments*. (Kushner DE ed) Academic Press, London, pp. 318-368.

Laplace JM, Delgenes JP, Moletta R and Navarro JM (1993). Ethanol production from glucose and xylose by separated and co-culture processes using high cell density systems. *Proc. Biochem.* **28**: 519-523.

Laubinger W and Dimroth P (1988). *Biochemistry* **27**: 7531-7537.

Lonsane, B. K. and Ramesh, M. V. (1990). Production of bacterial thermostable a-amylase by solid state fermentation: a potential tool for achieving economy in enzyme production and starch hydrolysis. *Adv. Appl. Microbiol.* **35**, 1-56.

Mamo G (1996). Properties of a thermostable amylase from thermophilic *Bacillus* sp. WN11 and its production through immobilization. *M.Sc. Thesis*, Addis Ababa University, Addis Ababa.

Mamo G and Gessesse A (1997). Thermostable amylase production by immobilised thermophilic *Bacillus sp. Biotechnol. Tech.* **11**: 447-450.

Mattiasson, B (1986). Technological processes for biotechnological utilization of microorganisms. In: *Biotechnology: potentials and limitations* (Silver S ed), pp. 113-125, Springer Verlag, Berlin.

Mattiasson, B. (1983a). *Immobilized cells and organelles*. Vol. I. CRC Press, Florida, Boca Raton.

Mattiasson, B. (1983b). *Immobilized cells and organelles*. Vol. II. CRC Press, Florida, Boca Raton.

McCoy M (1998). Enzymes emerge as big ag feed supplements. *Chem. Eng*. News. May 4, 29-30.

Miller L (1959). Use of dinitrosalcylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.

Mitsuishi Y, Yamanobe T, Yagisawa M, and Takasaki Y (1987). Purification and properties of thermostable xylanases from mesophilic fungus strain Y-94. *Agric. Biol. Chem.* **51**: 3207-3213.

Moore JC and Arnod FH (1996). Directed evolution of a para-nitrobenzyl esterase for aqueous-organic solvents. *Nature Biotechnol.* **14**: 458-467.

Moreau AF Shareck, Kluepfel D and Morosoli R (1994). Increase in catalytic activity and thermostability of the xylanase A of *Streptomyces lividans* 1326 by site-specific mutagenesis. *Enzyme Microb Technol* **16**: 420-424.

Muller W, Winnefeld A, Kohls O, Scheper T, Zimelka W and Baumgarti H (1994). Real and pseudo oxygen gradients in Ca-alginate beads monitored during polarographic pO2 measurments using PT-needle microelectrodes. *Biotechnol. Bioeng*. **44**: 617-625.

Nakamura S, Wakabayshi K, Nakai R, Aono RR, and Horikoshi K (1993). Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M-1. *Appl. Env. Microbiol.* **59**: 2311-2316.

Nakamura S, Ishiguro Y, Nakai Y, Wakabayashi R, Aono, R., Horikoshi K. (1995). Purification and characterization of a thermophilic alkaline xylanase from thermoalkaliphilic *Bacillus* sp. strain TAR-1. *J. Mol. Cataly.B: Biocataly.* **1**: 7-15.

Nilsson, K., Birnbaum, S. and Flygare, S. (1983).A general method for the immobilization of cells with preserved viability. *Eur. J. Appl. Microbiol. Biotechnol.* **17**, 319-326.

Okazaki W, Akiba T, Horikoshi K, and Akahoshi R (1985). Purification and characterisation of xylanases from alkaliphilic thermophilic *Bacillus* sp. *Agric. Biol. Chem.* **49**: 2033-2039.

Olsson L and Hahn-Hagedal B (1993). Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates. *Proc. Biochem.* **28**: 249-257.

Omar SH (1993).Oxygen diffusion through gels employed for immobilization. *Appl. Microbiol. Biotechnol.* **40**, 1-6.

Pace NR (1997). A molecula view of microbial diversity and the biosphere. *Science* **276**: 734-740.

Page N, Kluepfel D, Shareck F, and Morosoli R (1996). Increased xylanase yield in *Streptomyces lividans*: dependence on number of ribosome-binding sites. *Nature Biotechnol.* **14**: 756-759.

Paice MG and Jurasek L (1984). Removing hemicellulose from pulps by specific enzymatic hydrolysis. *J. Wood Chem. Technol.* **4**: 187-198.

Pandey A (1992). Recent process developments in solid state fermentation. *Proc. Biochem.* **27**, 109-117

Park YS, Yum DY Bai DH and Yu JH (1992). Xylanase from alkaliphilic *Bacillus* sp. YC-335. *Biosci. Biotech. Biochem.* **56**: 1355-1356

Pen J, Verwoerd TC, Vanparidon PA, Beudeker RF, Vandenelzen PJM, Vanderklis JD, Versteegh HAJ, Vanooyen AJJ and Hoekema A (1993). Phytase-

containing transgenic seeds as a novel feed additive for for improved phosphorus utilization. *Biotechnology* 11: 811-814.

Pere J, Siika-aho M, Buchert J and Viikari L (1995). Effect of purified *Trichoderma reesei* cellulases on the fiber properties of kraft pulp. *Tappi J.* 78, 71-78.

Poutanen K (1997). Enzymes: an important tool in the improvement of the quality of cereal foods. *Trends Food Sci. Technol.* 8: 300-306.

Prior BA, Kilian SG, and du Preez, JC (1989). Fermentation of D-xylose by the yeasts *Candida shehatae* and *Pichia stipitis*: prospecs and problems. *Proc. Biochem.* **24**: 21-32.

Ramakarishna SV, Suseela T, Ghildyal NP, Jaleel SA Prema P, Lonsane BK and Ahmed SY (1982). Recovery of amyloglucosedase from moldy bran. *Indian J Technol.* **20:** 476-480.

Ramesh MV and Lonsane BK (1987). Solid state fermentation for production of α-amylase by *Bacillus megaterium* 16M. *Biotechnol. Lett.* 9, 323-328.

Ramesh MV and Lonsane BK (1989). Solid state fermentation for production of higher titres of thermostable *alpha*-amylase with two peaks for pH optima by *Bacillus licheiformis* M27. *Biotechnol. Lett.* 11, 49-52.

Ramsay JA, Hassan MCA and Ramsay BA (1998). Biological conversion of hemicellulose to propionic acid. *Enz. Microb. Technol.* **22**: 292-295.

Ratto M, Poutanen K, and Viikari L (1992). Production of xylanolytic enzymes by an alkalitolerant *Bacillus circulans* strain. *Appl. Microbiol. Biotechnol.* 37: 470- 473.

Rehm HJ and Omar SH (1993). Special morphological and metabolic behaviour of immobilized microorganiasms. In: *Biotechnology*,2nd ed. Rhem, H. J. and Reed, G. (eds), Vol.1, pp: 223-248, Weinheim: VCH Verlagsgesellschaft.

Reichelt JR (1983).). In: *Industrial enzymology: the application of enzymes in industry.* (Godfrey T and Reichelt JR, eds.) Macmillan Publ. Ltd. Basingstoke, pp. 210-220.

Richter G, Cyriaci G, Petzold A and Schwartze J (1990). The effect of the enzyme preparation Endofeed in the feeding value of triticale to broilers. *Arch. Anim. Nutr.* 40: 959967.

Senior DJ, Hamilton J, Bernier RL, and du Manoir JR (1992). Reduction in chlorine use during bleaching of kraft pulp following xylanase treatment. *Tappi J.* **75**: 125-130.

Shinmyo A, Kimura H and Okada H (1982). Physiology of *alpha*-amylase production by immobilized *Bacillus amyloliquefaciens*. *Eur. J. Appl. Microbiol. Biotechnol.* 14: 7-12.

Shoham Y, Schwartz Z, Khasi A, Gat O, Zosim Z, and Rosenberg E (1992). Delignification of wood pulp by a thermostable xylanase from *Bacillus stearothermophilus* strain T-6. *Biodegradation* **3**: 207-218.

Singh A, Goel R, and Johri BN (1990). Production of cellulytic enzymes by immobilized *Sportrichum themophile*. *Enzyme Microb. Technol.* **12**, 464-468.

Smith DC and Wood TM (1991). Isolation of mutants of Aspergillus awamori with enhanced production of extracellular xylanase and B-xylosidase. *World J. Microbiol. Biotechnol.* 7: 343-354.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, and Klenk DC (1985). Measurement of protein using bicinchonic acid. *Anal Biochem.* **150**: 76-85.

Sneath, PHA, NS Mair, ME Sharpe and JG Holt (1986). *Bergey's Manual of systematic Bacteriology*. Vols 2-4, Wiliams and Wilkins, London

Solis-Pereira S, Favela-torres E, Viniegra-Gonzalez G and Gutierrez-Rojas M (1993). Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentation. *Appl. Microbiol. Biotechnol.* **39**, 36-41.

Sugiyama S (1995). Na^+-driven flagellar motors as a likely Na^+ re-entry pathway in alkaliphilic bacteria. *Mol. Microbiol.* **15**: 592

Sunna A, and Anthranikian G (1996). Growth and production of xylanolytic enzymes by the extreme thermophilic anaerobic bacterium *Thermotoga thermarum*. *Appl. Microbiol.. Biotechnol.* **45**: 671-676.

Sunna A, Puls J, and Antranikian G (1996). Purification and characterization of two thermostable endo-1,4-β-D-xylanases from *Thermotoga thermarum*. *Biotechnol. Appl. Biochem.* **24**: 177-185.

Talling JF, Wood RB, Prosser MV, and Baxter RM (1973). The upper limit of photosynthetic productivity by phytoplankton: evidence from Ethiopian soda lakes. *Freshwat. Biol.* 3: 53-76.

Tanaka H, Ohta T, Harada S, Ogboona JC and Yajima M (1994). Development of fermentation method using immobilized cells under unsterile condition. 1. Protection of immobilized cells against antimicrobial substances. *Appl. Microbiol. Biotechnol.* **41**, 544-550.

Teather RM and Woo PJ (1982). Use of congo red-polysaccharide interactions in enumeration and characterisation of cellulolytic bacteria from the bovine rumen. *Appl Env Microbiol* **43**: 777-780.

Thomson JA (1993). Molecular biology of xylan degradation. *FEMS Microbiol. Rev.* **104:** 65-82.

Tobey JF and Yousten AA (1976). Factors affecting the production of amylase by *Bacillus thuringiensis*. *Dev. Ind. Microbiol.* **18:** 499-510.

Tonkova A, Ivanova V, Dobreva E, Stefanova M, and Spasova D (1994). Thermostable α-amylase production by immobilized *Bacillus lichiniformis* cells in agar gel and on acrlonitrile/acrylamide membranes. *Appl. Microbiol. Biotechnol.* **41**: 517-522.

Tsujibo H, Sakamoto T, Nishino N, Hasegawa T, and Inamor Y (1990). Purification and properties of three types of xylanases produced by an alkaliphilic actinomycete. *J. Appl. Bacteriol.* **69**: 398-405.

Uffen RL (1997). Xylan degradation: a glimpse at microbial diversity. *J. Ind. Microbiol. Biotechnol.* 19: 1-6.

Viikari L, Kantelinen A, Sundquit J and Liko M (1994). Xylanases in bleaching: from an idea to the industry. *FEMS Microbiol. Rev.* **13**: 335-350.

Wikie KCB (1983). Hemicellulose. *CHEMTECH.* **13**: 306-319.

Winterhalter C, and Lieb W (1995). Two extremely thermstable xylanases of hyperthermophilic bacterium *Thermotoga maritima* MSB8. *Appl. Env. Microbiol.* **61**: 1810-1815.

Wong KKY and Saddler JN (1993). Application of hemicellulases in the food, feed, and paper industries. In: *Hemicellulose and Hemicellulases*. Coughlan MP and Hazlewood GP, eds. Portland Press, London. pp. 127-144.

Wong KKY, Martin LA, Gama FM and Saddler JN (1997). Bleach boosting and direct brightening by multiple xylanase treatments during peroxidase bleaching of kraft pulps. *Biotechnol. Bioeng*. **54**: 312-318.

Wong KKY, Tan LUL, and Saddler JN (1988). Multiplicity of β-1,4- xylanase functions and applications. *Microbiol. Rev*. **52**: 305-317.

Wood, RB and Talling JF (1988). Chemical and algal relationships in a salinity series of Ethiopian inland waters. *Hydrobiologia* **158**: 29-67.

Yang VW, Zhuang Z, Elegir G and Jeffries TW (1995). Alkaline active xylanase produced by a alkaliphilic Bacillus sp. isolated from kraft pulp. *J Ind. Microbiol*. **15**: 434-441.

Zamost BL, Nielsen HK and Starnes L (1991). Thermostable enzymes for industrial applications. *J. Ind. Microbiol*. **8**: 71-82.

Zhilina TA, Zavarzin GA, Raney F, Kevbrin VV, Kostrikina NA, and Lysenko (1996). *Spirochaeta alkalika* sp. novo., *Spirochaeta africana* sp. novo., and *Spirochaeta asiatica* sp. novo. alkaliphilic anaerobes from the continental soda lakes in central Asia and the East African Rift. *Int. J. Syst. Bacteriol*. **46**: 305-312.