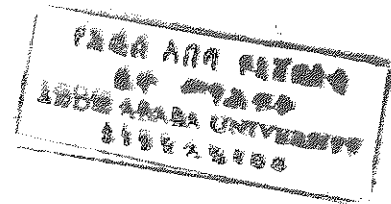


ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES

Thermastable and alkaline xylanase from an alkaliphilic Actinomycete

By
Amare Siraw



A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

Approved by Examining Board:

Dr Amutha R. (Examiner)

Dr Fasil Assefa (Examiner)

Dr Dawit Abate (Advisor)

Dr Amare Gessesse (Advisor)

Dr Dawit Abate (Chairman)

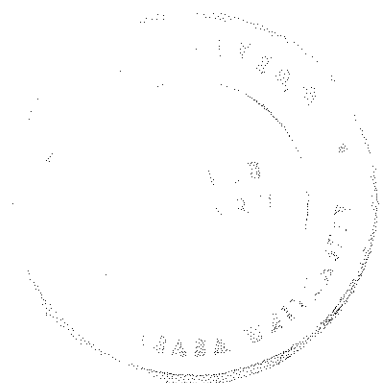
R. Assefa

F. Assefa

D. Abate

A. Gessesse

D. Abate



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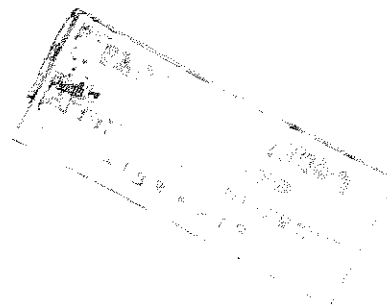
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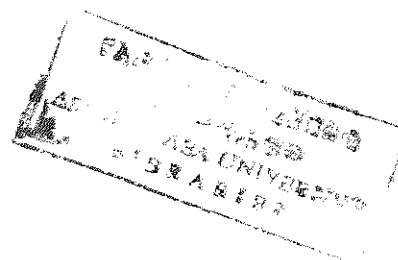
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Abbreviations

CMC	Carboxymethyl Cellulose
DNS	Dinitro salicylic acid
NSP	Non-starchy Polysaccharides
RT	Room Temperature
SSF	Solid- State Fermentation
TCA	Trichloroacetic Acid



Abstract

A xylanase producing alkaliphilic actinomycete strain designated AS-19 was isolated from Lake Abjata, an alkaline Soda Lake in Ethiopia. The strain produce xylanase in solid state fermentation (SSF) using different agricultural residues (wheat bran, sugarcane bagass, and saw dust) as substrates without enrichment of the medium .The highest level of enzyme was produced in wheat bran (165 U/g). Maximum production of xylanase was observed at wheat bran-to-moisturing agent ratio of 1:1.5(w/v) at 37 °C for 72 h. Birch wood xylan enhances enzyme production with 110 % while xylose, arabinose, glucose, lactose and fructose strongly repressed the enzyme production. In the presence of metal ions such as HgCl₂, and, ZnSO₄ the activity of the enzyme was strongly inhibited. Birch wood xylan was hydrolyzed more rapidly than oat spelt xylan. The optimum pH of the enzyme was 8.5-10 and stable at alkaline pH (8-10). The optimum temperature for enzyme activity was 70-80 °C at pH 9. The enzyme after 4 h incubation retained 77.7 and 54 % of its original activity at 75 and 80 °C respectively, at pH 9. The enzyme is also stable over wide temperature range (50-80 °C) for 1 h at pH 9. Thus, the result showed that the enzyme is both alkaline and thermostable. The enzymatic products of xylan hydrolysis were a series of short-chain xylooligosaccharides, indicating the enzyme was an endoxylanase. These are some characteristics that make this enzyme potentially very attractive for industrial applications.

Key words: *Actinomycete; xylan; xylanase; alkaliphile.*

1. Introduction

1.1. Hemicelluloses

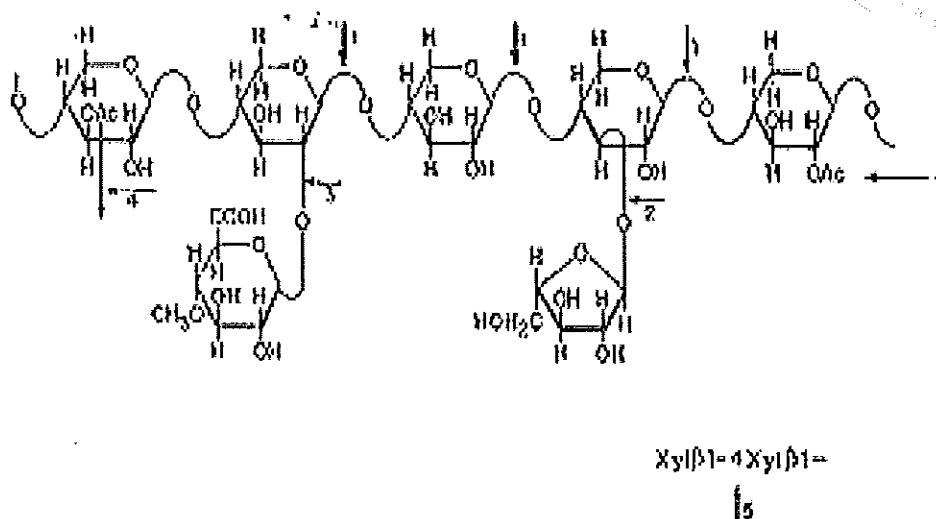
The term hemicellulose represents a heterogeneous group of plant cell wall polysaccharides which are linear or branched polymers, easily hydrolyzed by mineral acids, and give a variety of sugars up on hydrolysis (Suurnakki *et al.*, 1997). Xylan, having a β -1, 4-linked xylopyranoside residues as its backbone, is the major component of hemicelluloses. Xylan is a heteropolysaccharide usually having a branched chain. Branching of L-arabinofuranose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid at C₂ and C₃ positions of the D-xylose residue are very common (Figureure 1).

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 through hydrogen bonding (Eriksson *et al.*, 1990). This structural arrangement is believed to be important in maintaining the integrity of cellulose and protects the fibers from attack by cellulases (Buchert *et al.*, 1994).

1.2 Enzymes involved in the hydrolysis of xylan

Hydrolysis of xylan to its constituent sugars is brought about by different enzymes which act cooperatively (Figureure 1). The enzyme involved in the hydrolysis of the internal β -1, 4-xylopyranosidic linkage is known as endoxylanase, which randomly attacks the internal β -1, 4-linkage. The main products of the reaction catalyzed by endoxylanase are xylooligosaccharides of different chain length. Xylooligosaccharides thus produced are acted upon by β -xylosidase to produce xylose (McCleary 1986; Poutanen and Puls, 1988). Both xylanase and β -xylosidase are

not capable of hydrolysing the side chain sugars at the C₂ and C₃ position of xylose residues of xylan. This function is therefore carried out by side chain hydrolysing enzymes. These are α-glucuronic acid and 4-O-methyl glucuronic acid side chains; α-arabinofuranosidase that 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 (Cesar and Marsa, 1996; Singh *et al.*, 2000).



- 1 ENDO-1,4-β-XYLANASE (EC 3.2.1.8)
- 2 α-L-ARABINOFURANOSIDASE (EC 3.2.1.33)
- 3 α-GLUCURONIDASE (EC 3.2.1)
- 4 ACETYL ESTERASE (EC 3.1.1.6)
- 5 β-XYLOSIDASE (EC. 3.2.1.37)

Figure 1. Structure of xylan and enzyme cleavage sites

1.3 Microbial sources of xylanase production

To date, diverse genera and species of bacteria and fungi are becoming rich source of xylanase. However, the enzymes from different sources differ in their temperature and pH activity (Table 1-2).

Table 1. Characteristics of some xylanases from fungi

Microorganisms	Optimal Temperature (° C)	Optimal pH	Reference
<i>Aspergillus aculeatus</i>	50	4.0	Fujimoto <i>et al.</i> ,1995
<i>Aspergillus nidulans</i> kk-99	55	8.0	Taneja <i>et al.</i> ,2002
<i>Aspergillus oryzae</i>	60	5.0	Kitamoto <i>et al.</i> ,1999
<i>Aspergillus terreus</i>	50	7.0	Ghanen <i>et al.</i> ,2000
<i>Fusarium oxysporum</i>	55-60	6.0	Christako <i>et al.</i> ,1996
<i>Melanocarpus albomyces</i>	70	7.0	Jain <i>et al.</i> ,1998
<i>Thermoascus aurantiacus</i>	70-75	4-4.5	Kalogeris <i>et al.</i> ,1998

1.4 Application of xylanase

Xylanases have potential application in a wide range of industrial processes. Some of the major applications of xylanase are summarized below.

1.4.1 Application of xylanase in enzyme-assisted kraft pulp bleaching

Pulp for paper and paperboard is conventionally processed by kraft pulping. In this process, the first step 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 (Viikari *et al.*, 1994). The remaining 5-10% lignin is highly modified under the process of cooling thus giving the characteristics black or brown color of kraft pulps. To produce high quality white paper, a series of bleaching steps, which involve elemental chlorine, are carried out to remove the residual modified lignin (Patel *et al.*, 1993). Although chlorine-based bleaching of pulps is effective in removing the residual lignin, it results in chlorinated organic by products which are reported to have highly persistent toxic and mutagenic effects (Srinivasan and Rele, 1999; Techapun *et al.*, 2002). Because of the growing public concern about environment and strict legislations regarding pollution, the search for alternative ways to reduce or avoid the release of chlorogenic compounds with kraft mill bleaching effluent has been promoted.

Enzymatic treatment of kraft pulp prior to bleaching hydrolyses the xylan component of wood, to facilitate lignin removal. Consequently, it has shown a substantial reduction in the use of chlorine for subsequent bleaching operations (Viikari *et al.*, 1994). It has also been shown that xylanase treatment is important in achieving the necessary brightness of pulp in a total chlorine free bleaching operations (Paice *et al.*, 1988; Patel *et al.*, 1993). Xylanases from different organisms have been evaluated for their interaction with various kinds of pulps. On the laboratory scale,

xylanases from *Streptomyces roseisc eroticus* and other actinomycetes have been used for enzymatic pulp treatment to test their bleach boosting abilities and showed an increase in brightness by 2-5 % (Patel *et al.*, 1993).

The interests in xylan degrading enzymes and their application in the pulp and paper industries have increased significantly over a past few years (Gupta *et al.*, 2000). In the kraft process of pulp production, the pulp prior to the normal bleaching operation has an alkaline pH and high temperature (Roncern *et al.*, 2005). Thus, xylanase active and stable at high temperature and alkaline range are very important in the process of enzymes-assisted kraft pulp bleaching. Most xylanase known to date are optimally active at temperature below 50 °C and act in acidic or neutral pH (Blanco *et al.*, 1995). Therefore, the use of such xylanase 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. This process of cooling and reheating, and lowering and raising the pH of the large pulp mass is very expensive, taking considerable time, energy and chemicals. This in turn discourages many pulp and paper mills to use enzymes based pulp bleaching (Kulkarni *et al.*, 1999).

The use of alkaline active xylanases allows direct enzymatic treatment of the alkaline pulp and avoids the cost incurring and time consuming steps of pH re-adjustment. In particular, alkaline xylanases, which are operationally stable at higher temperature, are more beneficial because of savings in cooling cost and time



1.4.2 Xylanase in Baking industry

The main components of non-starch polysaccharides in wheat flour are pentosans (mainly arabino xylans, AX). Arabinoxylans occur as minor components of wheat flour (2-3%) on dry basis, and can be divided into soluble water-extractable arabinoxylans (WE-AX) and insoluble water-unextractable arabinoxylans (WU-AX). However, they play an important role in dough rheology and bread quality (Courtin and Delcour, 2002). Xylanase can randomly attack the arabinoxylan backbone to cause a decrease in the degree of polymerization, hence have a strong impact on the structure and function of arabinoxylan. Xylanases may be used to accelerate the baking of bread and other foods by helping to break down polysaccharides in dough (Courtin and Delcour, 2002).

It has been described that enzyme hydrolysis of non-starch polysaccharide led to the improvement of rheological properties of dough, bread specific volume, and crumb firmness (Martinez-Anaya and Jimenez, 1997). There is currently much interest in using non-starch polysaccharides hydrolyzing enzymes (especially xylanases) in bakeries. Xylanases from *Aspergillus niger* var. *awamori* improved the quality of bread by 30 % increase in the specific bread volume (Norma and Guillermo, 2003). This is further enhanced when amylase in combination with the xylanase is used (Vardakou *et al.*, 2003). Knowledge about the mechanisms of xylanase action in dough and bread is still limited, probably because the content of pentosans in flour varies amongst wheat types, and specificity of xylanases to wards different substrates is also variable (Vardakou *et al.*, 2003). While many researchers have investigated the effects of the mesophilic xylanases on bread making; comparatively little has been done on the thermo stable xylanases (Courtin and Delcour, 2002).

1.4.3 Xylanases in Animal Feed industry

Xylanases have been successfully used in monogastric diets to hydrolyze non-starchy polysaccharides (NSP) such as arabinoxylans (Walsh *et al.*, 1993). The presence of high levels of NSP in cereal-based diet results in poor feed conversion rate, slow weight gain, and sticky droppings by young animal, especially chicks (Baker and Wicker, 1996; Perry *et al.*, 1996). Addition of β -glucanases and xylanases during feed production was found to degrade NSP and markedly improve the digestion and absorption of feed components as well as weight gain by broiler chickens and egg laying hens (Walsh *et al.*, 1993).

Similarly, there is a great deal of interest in using enzyme preparations containing high levels of cellulase and hemicellulase activities for improving the feed utilization, milk yield and body weight gain by ruminants. Beauchemin *et al.*, (1995) reported that addition of commercial enzyme preparations containing cellulase and xylanase to hay diet increased the live weight gain of cattle by as much as 35%. Similarly, a 5-25% increase in milk yield has been reported in the case of dairy cows fed with forage treated with commercial fibrolytic enzymes (Lewis *et al.*, 1996).

1.4.4 Other applications of xylanases

Xylanase also find potential applications in several industrial processes. Along with pectinase and cellulases, xylanase play a key role in maceration of vegetable matter (Beck and Scoot, 1974), clarification of juices and wine (Biely, 1985), extraction of flavors, plant oils (McClary, 1986), for bioconversion of lignocelluloses to sugar, ethanol, and other useful substances (Polizeli *et al.*, 2005).

1.5 Solid State Fermentation

Xylanases can be produced using two main methods, solid-state cultivation systems and submerged liquid cultivation systems. Solid- State Fermentation (SSF) is defined as the fermentation involving solids in the absence (or near absence) of free water. The substrate however, must possess enough moisture to support growth and metabolism of the microorganisms (Pandey, 1992). SSF system offers numerous advantages including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipment, lower capital investment and lower operating cost (Holker and Jurgen, 2005). SSF process is especially ideal for developing countries. Since it uses agro-industrial residues that are cheaper source of substrates. Some of the substrates that have been used include sugar cane bagass, wheat bran, rice bran, saw dust, corncobs, banana waste, tea waste etc (Pandey *et al.*, 1999).

The selection of a substrate for enzyme production in a SSF process depends up on several factors, mainly related with cost and availability of the substrates that necessitates screening of several agro- industrial residues (Pandey, 2003). In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it and serve as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such case, it would become necessary to supplement them externally with nutrients. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using them in SSF processes (e.g.,

lignocellulose), there by making them more easily accessible for microbial growth (Pandey *et al.*, 1999).

The major factors that affect microbial synthesis of enzymes in a SSF system include; selection of a suitable substrate and microorganism, pre-treatment of the substrate, particle size (inter-particle space and surface area) of the substrate, water content and water activity of substrate, relative humidity, type and size of the inoculum, control of temperature of fermenting matter/removal of metabolic heat, period of cultivation, maintenance of uniformity in the environment of SSF system and gaseous atmosphere, i.e., oxygen consumption rate and carbon dioxide evolution rate(Pandey, 2003).

Although all the aforementioned factors affect SSF system, water activity and particle size are by far the most important ones to influence the process (Holker and Jurgen, 2005). Smaller substrate particle provide larger surface area for microbial attack, but too small a substrate particle may result in substrate agumulation, which may interfere with microbial respiration/aeration, that results in poor growth. On the other hand, larger particles provide better respiration/aeration efficiency (due to increased inter- particle space), but provide limited surface for microbial attack. This necessitates a compromise particle size for a particular process. Although SSF found to be suitable the growth of fungi that needs relatively lower water activity, there are many reports that indicate that bacteria also perform well in SSF system (Virupakshi *et al.*, 2005; Khandeparkar and Bhosle, 2006).

1.6 Searching of xylanase of potentially importance

Most xylanase studied to date are optimally active at, or near, mesophilic temperatures (approximately 40-60 °C) and neutral pHs (Belancie *et al.*, 1995; Khandeparkar and Bhosle, 2006). However, the application of xylanases in recent years calls for there perform at extreme conditions. The chemical and functional stability of these enzymes towards these environments are important for their industrial applicability. For example, in enzyme-assisted pulp bleaching operations, xylanases having optimum activity and stability at elevated temperature and alkaline pH offer tremendous technical and economical advantages (Viikari, *et al.*, 1994).

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

Though significant progress has been made in recent years in developing and perfecting protein-engineering techniques through site directed mutagenesis and random mutagenesis, understanding of protein structure to function relationship is not still perfect and thus predictions made on the basis of structural information have in many occasions failed to bring about the desired result (Kuchner and Arond, 1997; Bloom *et al.*, 2005). Although the creation of disulfide cross links through protein engineering may prove useful for increasing the thermostability of xylanases for biotechnological processes, its use is restricted where disulfide bonds are stable. Interestingly, xylanases without S-S cross- links are known to be more thermostable than those with disulfide bonds (Oku *et al.*, 1993). Thermostability of the enzyme from hyperthermophiles also appears to be the result of the reduction of water-accessible hydrophobic surfaces rather than the disulfide cross-link strategy (Adams, 1993). Thus, even though protein engineering can be used to increase

conformational stability and enzyme activity, much remains to be learned from natural thermophilic enzymes before precise molecular predictions can be guaranteed.

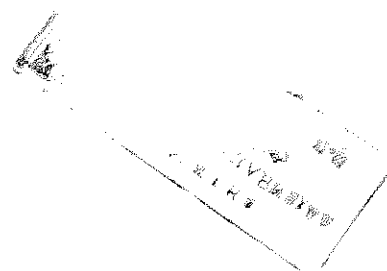
Given the dearth of information on application of protein engineering, isolation of new strains producing novel enzymes of potential in industrial importance from the environment may offer a better option. One potential source of industrially important xylanase is to isolate xylanolytic microorganisms from extreme habitats, so called extremophiles, which are characterized with high temperature, very alkaline or acidic environment etc. Moreover, expanding the potential application of biocatalysts requires the study of extremophiles and their enzymes to extend the present understanding of protein chemistry.

Though a lot of mesophilic microbial strains with optimum growth in the neutral pH range are known to produce alkaline active proteases and very thermo stable amylases, to date few thermo stable and alkaline xylanase has been reported from such organisms (Virupakshi *et al.*, 2005). To obtain thermostable xylanases many researchers focused their attention on the isolation of thermophilic microorganisms.

Consequently many xylanase producing thermophilic fungi, bacteria, and archea have been isolated in different laboratories (Coral *et al.*, 2002; Suren *et al.*, 2003; Sunna and Anthranikian, 1996). Though most of these organisms produce thermostable xylanases they showed optimum activity and stability at or below neutrality (Virupakishi *et al.*, 2005). Other studies were also directed to the study of xylanases from alkaliphilic microorganisms. Many of the alkaliphilic microorganisms studied have been found to produce xylanases with pH optima in the near neutral region but with relatively high activities being retained in alkaline conditions (Blanco *et al.*, 1995). Almost all xylanolytic alkaliphiles known so far were isolated from neutral soil and

almost all strains belong to the genus *Bacillus* (Subramniyan and Prema, 2000). However, a number of xylanases with more alkaline pH optima have also been isolated from soda lakes and one the most alkaliphilic xylanases reported to date is xylB from *Bacillus* sp. AR-009, which has a pH optimum of 9-10 (Amare Gessesse, 1998; Gashaw Mamo, 2006).

Similarly other alkaliphile producing alkaline proteases, amylases, of potential in industrial application were isolated from alkaline soda lakes in Ethiopia (Gashaw Mamo and Amare Gessesse, 1999; Amare Gessesse *et al.*, 2003; Gashaw Mamo *et al.*, 2006). This indicates the great potential of this environment for supporting novel microbial strains that produce enzymes of potential biotechnological importance. However, there is little information about the alkaliphilic actinomycetes with xylanolytic properties elsewhere and Ethiopia (Mehta *et al.*, 2005).



1.7 Objectives of the study

The objectives of the present study were:

- To isolate xylanase producing alkaliphilic actinomycetes from Lake Abjata and characterize the crude enzyme to determine potential application.
- To study the production of xylanase of actinomycete sp AS-19 in solid-state fermentation using agro wastes as substrate.
- To assess the production enzymes other than xylanase by actinomycetes sp AS-19 in solid-state fermentation.

2. Materials and Methods

2.1 The organisms and cultivation conditions

2.1.1 Isolation of actinomycetes

Water and sediment samples were collected from Lake Abjata, an alkaline soda lake in Rift Valley of Ethiopia, using sterile plastic tubes. Isolation and enumeration of actinomycetes were performed by dilution plate technique using Starch-Casein Agar medium (SCAM) (Georis *et al.*,2000 Georis *et al.*,2000) containing (g/l; soluble starch, 10; casein, 0.3; KNO₃, 2.0; NaCl, 2.0; K₂HPO₄, 2.0; MgSO₄.7H₂O,0.05; CaCO₃. 02; FeSO₄.7H₂O, 0.01; Agar, 15). One-gram of sediment or one ml of water was taken in 9 ml of sterilized distilled water and agitated vigorously. pH was adjusted to about 10.3 by adding sterile sodium carbonate solution to a final concentration of 1% (w/v) after autoclaving.

Different aqueous dilutions (10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁷) of the suspension were inoculated on to the SCAM plates. After gently rotating, the plates were incubated at 30 °C and 37 °C for 5 to 7 days. Selected colonies (rough, chalky etc) of actinomycetes were picked and further purified by repeated plating. Plates containing pure cultures were stored at 4 °C until further examinations.

All isolates were identified as actinomycetes based on their colonial morphology, color of hyphae, and color of aerial mycelium (Williams and Cross, 1971 and Michael, 1986).

2.1.2 Screening for xylanase production

All isolates were screened for xylanase production by growing them on xylan medium (Amare Gessesse, 1998) consisting of (g/l): xylan, 5; peptone, 5; yeast extract, 2; K_2HPO_4 , 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.1; and Na_2CO_3 , 10. For solid media 15g/l purified agar was added. 1% w/v sodium carbonate was sterilized separately and added to the rest of the medium after cooling to adjust the pH to 10.3. Pure cultures of actinomycetes were inoculated on to appropriate media and incubated for 5 to 7 days. Then, the plates were flooded with 0.1% aqueous Congo red Solution and washed with 1M NaCl (Teather and Woo, 1982). Xylanase producing colonies formed 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 (120 rpm at 37 °C) for three to five days. The level of xylanase production was determined from the cell free culture supernatant after centrifugation at 10,000g for 5 min.

2.1.3 Enzyme production in liquid culture (Submerged fermentation)

Fifty ml xylan medium in 250 ml flasks was inoculated with 2 ml of a 24 h fresh culture and incubated with rotary shaking (120 rpm at 37 °C) for three days. The Cell free supernatant obtained after centrifugation at 10,000g for 5 min was used as the enzyme source.

2.2 Solid State-Fermentation

2.2.1 Xylanase production in solid state culture

To 10g of wheat bran in Erlenmeyer flask (250 ml), stock mineral salt solutions were added to give a final salt concentration of (/g): K_2HPO_4 , 2.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.10 and $CaCl_2$, 0.050. Distilled water was added in such a way that the final wheat bran to moisture ratio was 1:1.5(w/w). After sterilization by autoclaving, the flasks were cooled and sterile Na_2CO_3 solution was added to give a final concentration of 1 % (w/w). The flasks were inoculated with 2 ml inoculum of an overnight culture or agar blocks with culture and incubated at 37 °C for 72 h. To study enzyme production using other substrates, sugar cane bagass and sawdust were used instead of wheat bran. The enzyme was extracted and assayed following standard assay condition (Miller, 1959).

2.2.2 Effect of moisture level on enzyme activity

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(w/w). All the liquid added into the flask was taken in to consideration in calculating the wheat bran to moisture ratio.

2.2.3 Effect of different carbon on xylanase production

The role of different additives on xylanase production was evaluated by supplementing 5 % (w/w) of different carbon with wheat bran. The enzyme was extracted and assayed from each set after incubation at 37 °C for 72 h.

2.2.4 Xylanase production with incubation period

A 10g sample of wheat bran was mixed with 15 ml of mineral salt solution in 250 ml flask, autoclaved, inoculated with 2 ml of an over night culture and incubated at 37 °C. 1% w/v sodium carbonate was sterilized separately and added to the rest of the medium after cooling to adjust the pH to 10.3. The contents of the flasks were harvested and assayed at 24 h interval.

2.2.5 Effect of incubation temperature on xylanase activity

The influence of temperature on the enzyme production by actinomycete strain AS-19 was studied by incubating at different temperature (Ambient Temperature, 30, 37 °C) for 72 h. 1% w/v sodium carbonate was sterilized separately and added to the rest of the medium after cooling to adjust the pH to 10.3.

2.2.6 Enzyme extraction

The enzyme from each flask was extracted using 100 ml distilled water. Each time the whole content was squeezed through a wet muslin cloth. The extract was centrifuged and the clear supernatant was used as the enzyme source.

2.3 Analytical techniques

2.3.1 Xylanase assay

Enzyme activity was assayed following the dinitro salicylic acid (DNS) method (Miller, 1959). To 0.9 ml of 1% birch wood xylan dissolved in 50 mM glycine-NaOH buffer (pH 9.0), 0.1ml of enzyme was added. After 15 min of incubation at 50 °C, the reaction was terminated by adding 2

ml of DNS solution and boiled for 5 min. Absorbance was measured at 540 nm against a reagent blank. One unit of xylanase activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar equivalent to xylose per minute. Xylanase production was expressed as U/g.

2.3.1.1 Effect of pH and temperature on xylanase activity: The effect of pH on xylanase activity was determined at various pHs using 50 mM citric-phosphate buffer (pH 4.0 to 6.0, 50 mM potassium phosphate buffer (7.0 to 7.5), 50 mM Tris -HCl buffer (8.0 to 8.5), and 50 mM glycine-NaOH buffer (9.0 to 10). To test the pH stability, the crude enzyme was diluted into the buffers described above and incubated for 1h at 50 °C. Residual enzyme (enzyme left over after 1 h) activity was then assayed at pH 9 as described previously.

The effect of temperature on xylanase activity was assessed by incubating under standard assay conditions the reaction mixtures at different temperatures in the range of 35 to 90 °C. Thermostability of the xylanase activity was monitored by incubating the enzyme sample for 1 h at various temperatures between 40 to 90 °C in 50 mM glycine -NaOH buffer (pH 9). The thermostability of xylanase was also monitored by incubating the enzyme sample for 4 h at temperatures of 75 and 80 °C; samples were withdrawn at 1h of intervals. The samples were kept at 4 °C until assayed for residual activity following standard assay procedure (Miller, 1959).

2.3.2 Cellulase assay

Cellulase activity was assayed using carboxymethyl cellulose (CMC) dissolved in 50 mM glycine- NaOH buffer, pH 9.0, as substrate (Miller, 1959). To 0.9 ml of CMC solution, 0.1 ml of appropriately diluted enzyme was added and incubated at 50 °C for 15 min. The reaction was

stopped by adding 2 ml dinitro salicylic acid (DNS) reagent followed by 5 min boiling. Absorbance was measured at 540 nm against reagent blank. One unit of cellulase activity was defined as the amount of enzyme that released 1 μ mol reducing sugar equivalent to glucose per minute. Cellulase production was expressed as U/g.

2.3.3 Protease assay

Protease activity was determined using casein as a substrate as described by Amare Gessesse and Berhanu Gashe (1997). The reaction mixture in a total volume of 2 ml was composed of 1% casein, 50 Mm glycien-NaOH buffer, pH 10, and appropriately diluted enzyme. After 30 min incubation at 50 °C, the reaction was terminated by adding equal volume of 10% trichloroacetic acid (TCA). After separation of the un-reacted casein precipitate by centrifugation, 0.5 ml of clear supernatant was mixed with 2.5 ml of 0.5M Na₂CO₃ and 0.5 ml of 1N Folin-Ciocalteau's phenol reagent. After 30 min at 25 °C, absorbance was measured at 660 nm against a reagent blank. One unit of protease activity was defined as the amount of enzyme that released 1 μ g amino acid equivalent to tyrosine per min under the standard assay conditions.

2.3.4 Amylase assay

Amylase activity was determined at 50 °C by mixing 0.9 ml of 1 %(w/v) soluble starch dissolved in 50 mM glycine-NaOH buffer, pH 9 with 0.1 ml o enzyme source. The reducing sugar released was measured following the dinitro salicylic acid (DNS) method (Miller, 1959). Absorbance was measured at 540 nm against a reagent blank. One unit of amylase activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar equivalent to glucose per minute under the assay condition. Amylase production was expressed as U/g.

2.3.5 Effect of metal ions on enzyme activity

The effect of metal ions on xylanolytic activity was tested by incubating the crude enzyme in the presence of 1 mM solution of MnCl₂, CuSO₄, Pb (CH₃) COO⁻, CaCl₂, ZnSO₄, MgSO₄, FeSO₄, KCl, NaCl, HgCl₂, FeCl₃, CoCl₂ for 30 minute at room temperature and then the residual enzyme was assayed following standard assay condition.

2.3.6 Thin layer chromatography (TLC)

For the chromatographic analysis of reaction products, 4 ml of 10-mg/ml birch wood xylan dissolved in glycine- NaOH buffer, pH 9, was mixed with 1 ml xylanase and incubated at 60 °C for 12 h. The hydrolysate was applied on to silica gel 60 F254 (Merck) and developed using butane-I-ol/ethanol/ water mixture (5:3:2 v/v) in 2 ascents, D-xylose as a standard. To develop spots the plate was sprayed with 30% (v/v) H₂SO₄ and heated at 100 °C.

Chemicals

Commercial xylans (birch wood xylan and oat spelt xylan) were purchased from sigma chemical co., USA. All other chemicals used in the present study were of highest purity grade. Materials such as wheat bran saw dust and sugar cane bagass were purchased from local markets.

Data presentations

All the experiments were performed in duplicate and the results are the mean of two values.

3. Results

3.1 The organisms

A total of 77 aerobic alkaliphile actinomycetes were isolated from Lake Abjata, 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. Twenty-seven strains (35%) formed detectable clear zone on solid media (Table 3). All xylanase positive isolates were grown in liquid xylan medium and the level of xylanase activity was determined from the cell free culture supernatant. A total of 11 isolates produced appreciable xylanase activity in liquid culture, out of which 6 produced xylanase activity greater than 1U. One strain, designated as AS-19 was selected for further study.

Table 3. A summary of results of xylanase screening on xylan containing solid Media.

Group	Xylanase production	Number of strains	%
1	+++	6	7.8
2	++	15	19.5
3	+	6	7.8
4	+/-	7	9.0
5	-	43	55.8

Key:

- +++ Excellent
- ++ Very good
- + Good
- +/- Not good
- No growth

3.2 Characterization of the enzyme

3.2.1 Effect of temperature on xylanase activity

The optimum temperature of the crude enzyme was determined on the isolate AS-19 by varying the reaction temperature at pH 9.0. The enzyme was optimally active between 70-80 °C (Figure 2). At 85 °C it retained about 90% of the optimum temperature while at 90 °C it retained 67%.

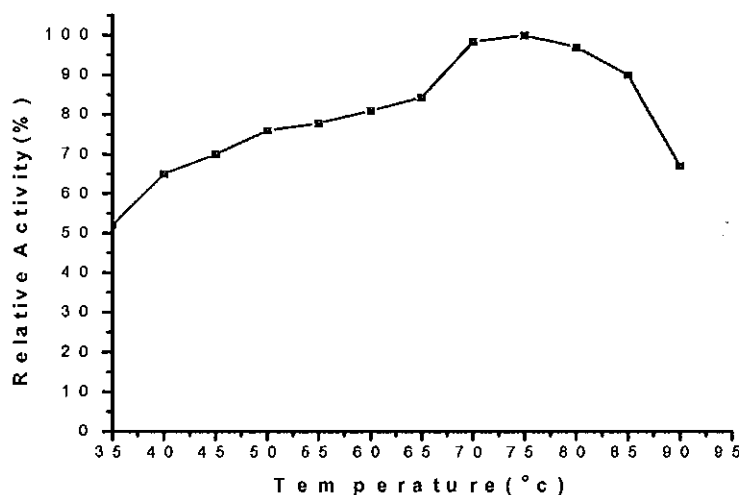


Figure 2: Effect of temperature on activity of xylanase. The temperature profile was determined by assaying Enzyme activity at different temperature values using 50mM glycine-NaOH buffer, pH 9

3.2.2 Effect of temperature on stability of xylanase

Thermostability of xylanase was determined by incubating the enzyme at different temperature in 50 mM glycine-NaOH buffer, pH 9 for 1h and residual activity was assayed using standard procedure (Figure 3a). The enzyme was stable at temperatures of 50 to 80 °C. At 90 °C, the enzyme retained 47.5% of its original activity.

The effect of temperature on stability was also determined by incubating the enzyme for 4h at 75 and 80 °C using pH 9 and pH 10 buffers. The enzyme showed good stability at both temperatures.

and pH values (Figure 3b). After 4 h incubation at 75 °C it retained 77.7 and 58.6% of its original activity at pH 9 and 10, respectively. After 4h at 80 °C it retained 54 and 58.7% of its original activity at pH 9 and 10 respectively. The enzyme showed almost similar stability at pH 10.

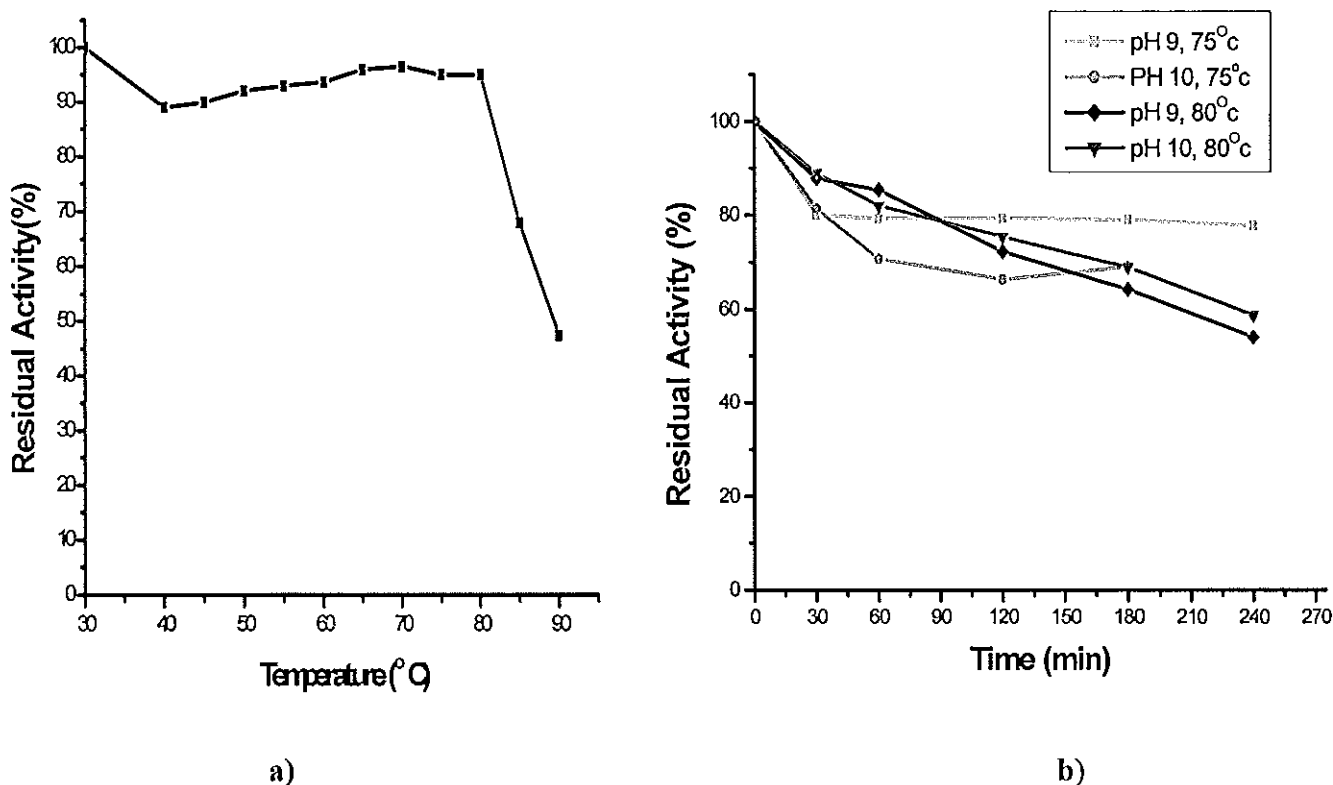
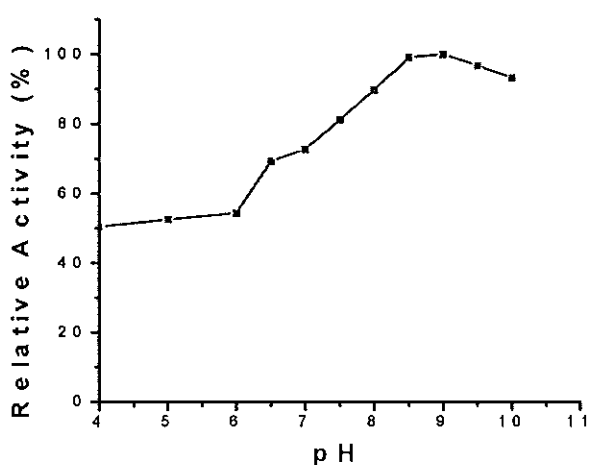


Figure 3: Effect of temperature on stability of xylanase. Effect of temperature on stability was tested by incubating the enzyme at different temperature values for 1h in 50mM glycine-NaOH buffer pH 9 and the residual activity was assayed at 50 °C following standard procedure.(Figure 3 a) .The enzyme was also incubated at 75 and 80 °C for 4 h at pH of 9 and 10. Samples were withdrawn at time interval and residual activity was measured following standard assay procedure (Figure3 b).

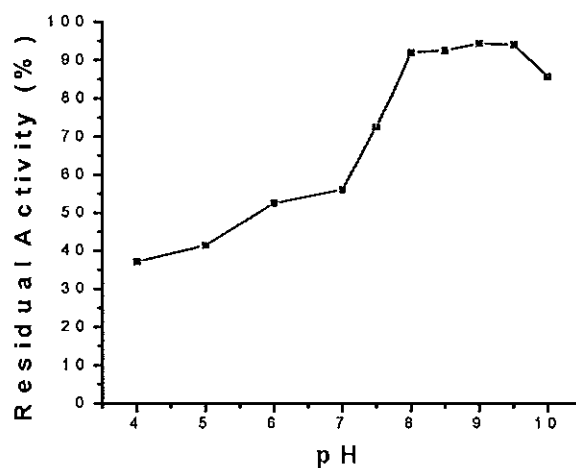
3.2.3 Effect of pH on activity and stability

The activity of xylanase at various pH values was measured by using birch wood xylan as the substrate at 50 °C at pH values ranging from 4 to 10. The xylanase showed to be active in a wide range of pH (Figure 4a). The optimum pH for activity was at pH 8.5 to 10, and over 70% of the peak activity was displayed between pH 7 and 10.

The effect of pH on stability was tested by incubating the enzyme at 50 °C for 1h in different buffers of varying pH values and residual activity was measured following standard assay procedure. The xylanase was stable at pH 8 to 10(Figure 4b).At pH 7, 56% of the original activity was retained.



a)



b)

Figure 4. Effect of pH on activity (a) and stability (b) of xylanase. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 1h at 50 °C and the residual activity assayed following standard assay procedure. Buffers used were: citrate-phosphate (4-6), Phosphate (7-7.5), Tris-HCl (8-8.5) and glycine-NaOH (9-10).

3.2.4. Effect of metal ions on xylanase activity

The effect of different metal ions on AS-19 xylanase was tested by assaying enzyme activity in the presence of different metal ions. Among those metal ions, HgCl₂ and ZnSO₄ showed strong inhibition while FeSO₄, MgSO₄, CaCl₂ and Pb (CH₃) COO⁻ resulted in partial inhibition (Table 4).

Table 4. Effect of metal ions on xylanase activity.

Compounds (Final Concentration 1 mM)	Relative activity (%)
Control(without metal ion)	100
MnCl ₂	77
CuSO ₄	86
Pb (CH ₃) COO ⁻	67
CaCl ₂	67.3
ZnSO ₄	47.2
MgSO ₄	63
FeSO ₄	61
KCl	99
NaCl	102
HgCl ₂	41
FeCl ₂	95
CoCl ₂	97.7

3.2.5 Enzyme mode of action

The hydrolysis products of birch wood xylan by xylanase were analyzed by thin layer chromatography. The hydrolysis products were X₂, X₃, X₄, X₅, X₆ and larger xylooligosaccharides (data not shown), indicating the xylan-binding xylanase was an endoxylanase.

3.2.6 Rate of substrate degradation

The effect of reaction time on enzyme activity was studied. The rate of substrate degradation by xylanase was rapid between 10 and 30 min and 20- 50 min was for birch wood and oat spelt xylan and attained its stationary phase after 30 min and 50 min, respectively (Figure 5). The comparative data revealed that the rate of hydrolysis was faster in birch wood xylan than oat spelt.

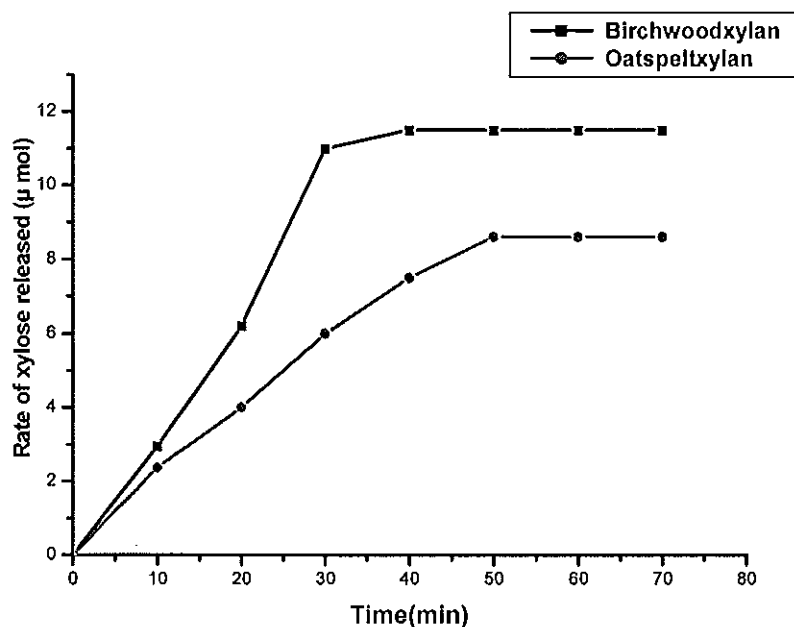


Figure 5: Rate of substrate degradation at pH 9.0 and at 50 °C.

3.3 Xylanase production by SSF of AS-19

3.3.1 Enzyme production

Actinomycetes sp AS-19 was grown both in solid and liquid medium. Compared to Submerged fermentation (112 U/ml) the level of enzyme production under SSF (180 U/ml) was very high. As indicated in Table (5), under SSF, in addition to xylanase the organism produced cellulase, and amylase but protease activity was not observed. The level of xylanase activity was high, while productivity of the other enzymes was low.

Table 5: Enzymes produced by actinomycete sp. AS-19 under SSF.

Enzyme	Activity (U/g)
Xylanase	144.3
Cellulase	37
Amylase	83
Protease	NA

NA: No activity.

3.3.2 Time course of xylanase production

The time course of enzyme production was studied using wheat bran as substrate. Enzyme production was followed for a period of 114 h (6 days). Maximum enzyme production was

observed from 24 h up to 72 h(138U/g). Further incubation after this time showed a gradual decline in the xylanase production (Figure 6).

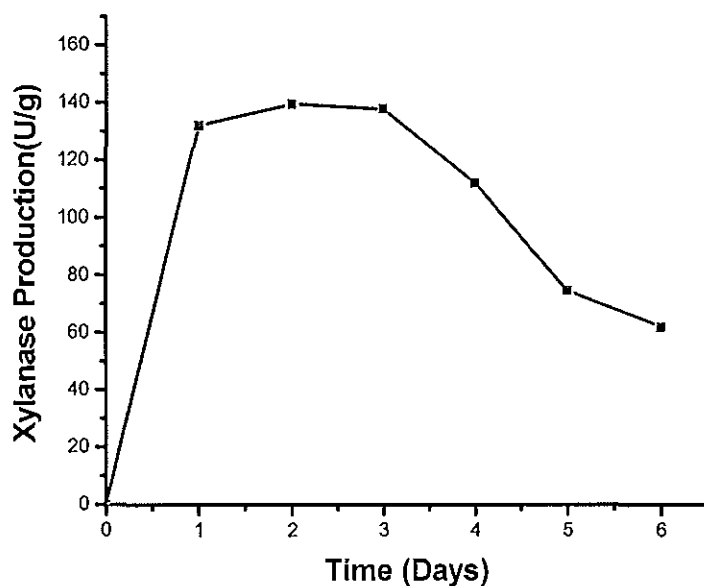


Figure 6: Time course of the xylanase production by actinomycete sp AS-19 on SSF.

3.3.3 Effect of incubation temperature on enzyme production

Enzyme production was affected by the incubation temperature. Maximum activity was detected at 30 °C Table (6). The amount of enzyme produced at Ambient Temperature 95.8 % of that produced at 30 °C.

Table 6. Effect of incubation temperature on enzyme production.

Temperature (°C)	Xylanase (U/g)
Ambient Temperature	138
30	144
37	130

3.3.4 Effect of moisture level on enzyme activity

Enzyme production was affected by the moisture content of the substrate. The highest xylanase production was observed in wheat bran-to-moisture ratio of 1:1.5. With increasing moisture level, enzyme productivity was decreased (Figure 7). At a ratio of 1: 2.5 and above less than 50% of the maximum xylanase production was observed.

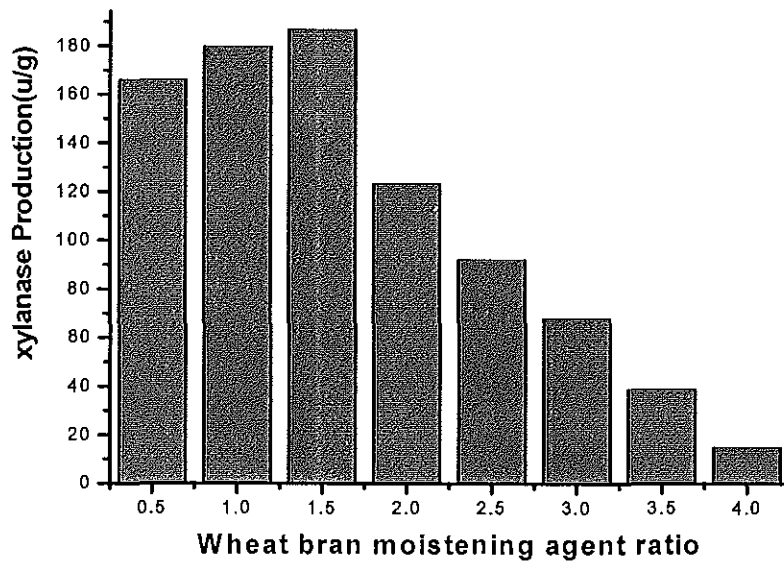


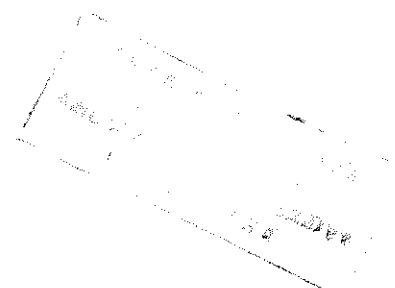
Figure 7: Effect of moisture level on xylanase production by actinomycete sp AS-19 under SSF

3.3.5 Effect of different carbon sources

In order to investigate the effect of sugars on the activity of xylanase, six different sugars were studied. Among the six different sugars tested, birch wood xylan was found to increase the enzyme activity slightly (110%), where as ariabinose, glucose, lactose and xylose were found to decrease the enzyme activity (Table 7). Oat spelt xylan showed no effect on the activity of AS-19 xylanase.

Table 7. Effect of various carbon sources on xylanase production

Carbon source	Relative activity (%)
None(with out additional carbon)	100
Ariabinose	36.7
Glucose	31.7
Lactose	50.9
Xylose	27.2
Birch wood xylan	110
Oat spelt xylan	100



3.3.6 Xylanase production on different substrate

Maximum xylanase production was observed when wheat bran was used as a substrate (Table 8). Enzyme production on sugar can bagass showed 83.5% of production on wheat bran. The lowest xylanase production was observed on saw dust substrate. Therefore, the comparative data revealed that sugar can bagass and saw dust produced lower xylanase than wheat bran.

Table 8. Effect of different substrate on xylanase production.

Nutrient sources	Xylanase(U/g)
Wheat bran	165
Sugarcane bagass	137.8
Saw dust	63

4. Discussion

In recent years a great deal of attention is given on alkaline active xylanases because of their potential application in different industrial processes especially in the process of enzyme – assisted pulp bleaching process (Roncern *et al.*, 2005). To date only few xylanolytic alkaliphiles are known. Most alkaline active xylanase producing organisms are isolated from neutral soil samples (Virupakshi *et al.*, 2005). Most xylanases, which are active and stable at alkaline pH and higher temperature, are obtained from microorganisms inhabiting extreme environments. Alkaline habitats are the sources of alkaliphiles and alkaline active enzymes.

Though only few studies on various enzymes have been conducted on Soda Lakes of Ethiopia (Amare Gessesse, 1998; Amare Gessesse *et al.*, 2003; Gashaw Mamo *et al.*, 2006), promising results were found on these Lakes. Novel enzymes produced from microbial strains, which could have a potential application in industry have been found from these Lakes.

In this study more than 27 isolates from Lake Abjata Soda Lake formed detectable xylanase activity of which strain AS-19 was found to produce better alkaline active xylanase. The Lake is highly productive (Amare Gessesse and Berhanu Gashe, 1997) it accommodates different groups of heterotrophic microorganisms that are expected to exist in the lake and be involved in the decomposition of various biological polymers thus playing a vital role in nutrient recycling. Although the great majority of xylanase producing alkaliphilic strains known so far belong to the genus *Bacillus* (Subramniyan and Prema, 2000), this study showed that alkaliphilic actinomycetes might also be important source of alkaline active xylanase.

In the kraft process of pulp production, the pulp prior to the normal bleaching operation has an alkaline pH and high temperature (Roncern *et al.*, 2005). Thus, xylanase active and stable at high temperature and alkaline range are very important in the process of enzymes-assisted kraft pulp bleaching. The xylanase isolated from AS-19 had an optimum activity (8-10)(Figure 1) and pH stability at 8.5-10 (Figure 1) and also optimally active (70-80 °C) (Figure 2) and stable for a wide temperature range (50-80 °C) (Figure 3). Thus, xylanase AS-19 might be functional to operate under conditions close to those of most mills, that is high pH (9-10) and temperature (70-75°C). So far only few xylanase with optimum temperature for activity exceeding 70 °C and above pH 9 have been reported (Amare Gessesse, 1998; Gashaw Mamo *et al.*, 2006; Khandeparkar and Bhosle, 2006).

Another interesting potential application for alkaline xylanase is in the hydrolysis of hemicellulosic biomass to simple sugars (Gupta *et al.*, 1992; Johnvesly *et al.*, 2002). Because xylan is soluble at alkaline pH, the high activity and stability of AS-19 xylanase at alkaline pH and relatively high temperature will be of great advantage for the hydrolysis of such wastes, especially in pulp and paper industry waste as it contains more dissolved xylan.

It is generally known that xylanases may be inhibited or activated by metal ions or other reagents. The activity of AS-19 xylanase was not significantly inhibited by the presence of different metal ions. However, it was strongly inhibited by HgCl₂ and ZnSO₄. A lot of impurities like metal ions, which can inhibit the activity of xylanase, exist in industrial wastes. The inhibition of the enzyme activity by HgCl₂ and ZnSO₄ ions may be due to its interaction with sulphhydryl groups, suggesting that there is an important cystein residue in or close to the active site of the enzyme.

Therefore, cloning of the gene may offer an additional advantage of improving this metal resistance properties of the enzyme through site-directed or random mutagenesis.

Among xylanolytic enzymes, endoxylanase are crucial for xylan depolymerisation and have received growing attention because of application in pulp and paper industries (Johnvesly *et al.*, 2002). The release of xylooligosaccharides from xylan by AS-19 xylanase supports its endo type mode of degradation.

When the effect of reaction time was investigated, it was observed that xylanase activity was maximum at 30 minute for birch wood xylan and 50 minute for oat spelt xylan (Figure 5). This might suggest that AS-19 xylanase do have high binding affinity to birch wood xylan than oat spelt xylan. There are reports on variation in incubation time for the determination of xylanase activity (Bucher *et al.*, 1994; Cesar and Mrsa, 196). As the enzyme –substrate interaction period increase, the amount of products also increase up to a certain point. Thus, understanding of the reaction time might assist to know when the maximum products occurred.

In addition to xylanase, the organism produced low level of cellulase and amylase activities but no protease activity was detected. The absence of protease is advantageous, because the xylanase AS-19 can maintain its stability during extraction and storage. In pulp and paper industry, cellulase is used together with xylanase to improve the drainage and runnability of the paper machines and to enhance the deinking of recycled fibers (Viikarie *et al.*, 1994; Johnvesly *et al.*, 2002).

Therefore, the existence of cellulase together with xylanase in strain AS-19 could have an important contribution during the operation of paper machines. However, the presence of high level of cellulase activity together with xylanase has been considered as a disadvantage in enzyme assisted pulp bleaching because of its adverse effect on pulp strength. In this aspect, overcoming cellulase activity in xylanase preparations is needed either using cloning and selective expression of xylanase genes in heterologous host systems or undertaking of site-directed mutagenesis. The presence of amylase together with xylanase could be seen as advantageous because amylase together with xylanase does have plenty of industrial applications such as in bakery and animal industries.



From the application point of view, faster production of an enzyme could be advantageous which may allow appreciable reduction in the production cost of the enzyme and products can be found in short period of time. Moreover, an organism that produces maximum xylanase activity in short incubation time offers significant advantage in reducing the risk of contamination. In this regard, the time required for maximum enzyme production by the strain AS-19 was short (1 to 3 days). Most studies have found maximum enzyme production after 4 days (Techapun *et al.*, 2001; Johnvesly *et al.*, 2002; Li *et al.*, 2006), though there were few that gave maximum enzyme production in 1-3 days (Georis, *et al.*, 2000; Fadel, 2001; Virupaksh, 2005).

The moisture content in SSF plays an important factor that determines the success of the process (Holker and Jurgen, 2005). Among the solid substrate-to-moisturizing agent tested for xylanase production by actinomycete sp AS-19, the maximum xylanase was obtained with wheat bran to water ratio of 1:1.5 (Figure 7). A decrease in enzyme production with an increase in moisture

level of the substrate could be attributed to a reduction in the degree of aeration with increasing moisture level. The role of moisture level in SSF media and its effect on microbial growth and biosynthesis of xylanases have been attributed to the impact of moisture on the physical properties of the solid substrate (Virupaksh, 2005).

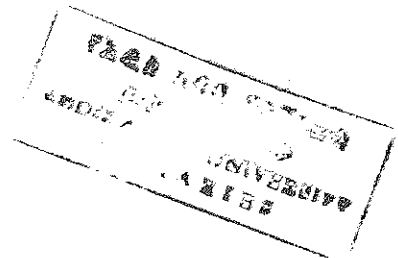
The moisture content beyond the optimum level inhibits enzyme production because the higher moisture levels decrease the porosity due to gummy texture of the substrate, alters the substrate particle structure, leads to poor oxygen transfer and decrease the diffusion (Pandy, 2003). The lower moisture level, than the optimum leads to the poor solubility of the nutrient of the solid substrate, improper swelling and a higher water tension (Pandy, 2003). In light of this fact, strain AS-19 needs medium solid-to-moisturizing agent for maximum xylanase production to do away with the effect by moisture content.

The incubation temperature affects the level of enzyme production under SSF (Holker and Jurgen, 2005). Actinomycte sp AS-19 showed optimum xylanase production at 30 °C and room temperature. Maximum production at lower temperatures may be advantageous because it can reduce the rate of evaporation during incubation. The fact that the strain produces maximum enzyme at room temperature hence facilitates the production of the enzyme with out the need of incubation instrument and reduce the cost of enzyme production.

One of the problems of producing enzymes for industrial application is the selection of cheap sources of substrate. This is because about 30-40% of the production cost is attributed to growth substrate (Kulkarni *et al.*, 1999). Thus, the use of low cost substrates for the production of industrial enzymes is one of the ways to greatly reduce production costs. To make the application

more cost effective at industrial level its production using low cost substrates like-agro wastes are very important. In light of this fact, wheat bran was compared with sugar cane bagass and saw dust. Comparing the above substrates with the largely used wheat bran, the results showed that for xylanase production sugar cane bagass or saw dust showed lower production than wheat bran. This difference in level of enzyme production could be attributed by many factors such as surface area, pretreatment, content and sugar composition of this substrate. But in area where wheat bran is not available bagass is the next replacement.

Among the different additives tested for xylanase production birch wood xylan induced the production of xylanase, while the rest of the sugars except oat spelt xylan, notably repressed the xylanase production which might be due to catabolite repression of the xylanase production. This implies wheat bran supplied enough nutrients without any need for addition of expensive supplements. This might be taken as an essential property of the strain AS-19. This is because maximum production of the enzyme was observed using non-expensive media that could highly reduce the cost of enzyme production. Many workers have reported the repression of xylanase production with the addition of different additives to solid substrate. The synthesis of xylanase by *Aspergillus niger* and *B.coagulans* on SSF was found to repressed by the addition of glucose/sucrose in the medium (Babu and Satyanarayana, 1993; Fadel, 2001).



5. Conclusion

1 Actinomycete sp AS-19 produces an alkaline xylanase, having low level cellulolytic activity.

The enzyme was active and stable up to 80 °C in alkaline solution (pH 9.0). The enzyme was also optimally active and stable in a broad pH range (8.5-10).

2 Strain AS-19 produced its maximum xylanase in wheat bran that used as substrate source. This implies wheat bran supplied enough nutrients without any need for addition of expensive supplements.

3 The strain grew in high pH value this can reduce the level of contamination, as few organisms are capable of growing under alkaline growth condition.

4 The strain grew in a short time of incubation. This is important to get the enzyme in very short time and thus reducing the cost of production and risk of contamination.

5 The strain also showed maximum enzyme production in lower temperature ranges.

This perhaps helps the strain AS-19 not to be affected by evaporation and produce the enzyme without any need of incubator and humidifier.

6 Resistance to high temperature and high pH are important characteristics in enzyme-assisted kraft pulp bleaching in paper and pulp processing because these conditions of temperature and pH are similar to those of pulp produced in paper industry. Consequently, the xylanase produced by actinomycete sp AS-19 may appear to be a candidate for use in the pulp and paper industry.

7 It is not easy to find an enzyme from organisms that suited entirely to particular application as it's from nature. Therefore, cloning of the gene may be essential to offer an additional or to improve some properties of the enzyme through site-directed or random mutagenesis. Therefore, it can be recommended that to enhance the catalytic efficiency, to inhibit cellulase activity and to make metal ions resistance of actinomycete sp AS-19 xylanase, using molecular manipulation or cloning of the gene coding for xylanase and expressing it in appropriate host is important.

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