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Master's Degree Thesis

Purification, characterization, and corroborated applications of xylanase from *Streptomyces* strains utilizing agro-waste

Graduate School of Chosun University

College of Pharmacy

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ABBREVIATIONS

BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CHAPS	3- [(3- Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CS628	*Streptomyces* sp CS628
CSWu-1	*Streptomyces* sp. CSWu-1
DMSO	Dimethylsulfoxide
EDTA	Ethylene glycol-bis (B-aminoethyl ether)-N, N, N' N'- tetraacetic acid
kDa	Kilo Dalton
K_m	Michaelis constant
NCBI	National Centre for Biotechnology Information
N- terminal	Amino (NH_2) terminal
PAGE	Polyacrylamide gel electrophoresis
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TEMED	N, N, N' N'- tetramethylethylenediamine
Tris	Tris [hydroxymethyl] aminomethane
V_{max}	Maximum velocity

국문 초록

Streptomyces 균주에서 xylanase 의정제, 특성분석 및 산업적 응용

라만 엠디 아리퍼

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최근 전라남도의 토양에서 두 방선균 *Streptomyces* sp. CS628 과 *Streptomyces* sp. CSWu-1 이 분리되었다. 이는 16s rDNA 와 rRNA 분석을 포함한 여러가지 실험을 통하여 그 특성이 분석되었다. 이 방선균들은 각각 Xyn628 과 XynWu-1 이라는 자일라나제를 생산하는데, 각각 다양한 배지에서 키워졌고, 겔 크로마토그래피법으로 분리 정제되고 생화학적 특성이 파악되었다. Xyn628 은 Sepharose CL-6B 컬럼을 이용한 겔 크로마토그래피로 한번에 분리되었고, XynWu-1 은 약한 이온교환크로마토그래피인 DEAE-sepharose 컬럼으로 정제한 후 Sephadex G-50 컬럼으로 최종 정제되었다. 각각의 분리능은 Xyn628 은 33.78%, XynWu-1 은 16 %이고, 각각 5.16 배와 4.87 배 농축되었다. 각 자일라나제의 분자량은 대략 18.1 kDa (Xyn628), 37 kDa (XynWu-1) 이다. 특히, Xyn628 은 보고된 방선균에서 분리한 자일라나제 중 가장 작은 분자량이다. N-terminal 시퀀싱의 결과 Xyn628 과 XynWu-1 은 각각 AYIKEVVSRAYM, AINVLVAAL 이다. 각각의 시퀀싱은 이들이 신규한 자일라나제임을 증명해준다. 이 둘은 매우 좋은 효소활성을 지녔고, 강한 알칼리 환경에서도 안정함을 보였다. 효소활성의 최적온도와 최적 pH 는 둘 다 60 ℃ and 11.0 이다. 이 두 자일라나제는 둘 다 pH 5.0 에서 pH13.0 까지의 환경에서 12 시간의 안정성을 보였고,

55 ℃ 에서 65 ℃ 에서 한시간 이상의 환경에서도 안정하였다. XynWu-1 의 효소 활성은 금속 이온이나 계면활성제에 의해서 크게 바뀐다. 특히 Mn^{2+} 이 효소 활성을 매우 크게 증가시키는 걸로 보아 이는 metalloprotein 이고 효소 반응의 촉매제로 사용될 수 있다. 두 자일라나제는 beechwood xylan 을 기질로 사용하였을 때, K_m 값은 각각 3.1 and 1.7 mg/mL 이었고, V_{max} 값은 각각 313 과 742 mmoL/min mg 이었다. Xyn628 은 자일로바이오스와 트라이오스를 생산하고, XynWu-1 은 자일로바이오스와 자일로테트라오스를 생산하는 것으로 보아 이 둘은 엔도자일라나제이다. SEM 을 통하여 보았을 때, 이 두 자일라나제는 옥수수대나 밀기울 등의 농업적쓰레기를 기질로 하여 분해할 수 있었다. 위 두 자일라나제는 내알칼리성, 내열성, 셀룰라이제에 안정함을 가지면서도, 매우 작은 분자량으로 매우 좋은 효소활성을 띈다. 이는 자일로올리고당 합성을 포함한 제지 표백공정, 바이오디젤 생산공정, 음식물쓰레기 처리공정, 프로바이오틱스의 용도 등의 다양한 산업적 응용을 기대할 수 있다.

ABSTRACT

Purification, characterization, and corroborated applications of xylanase from *Streptomyces* strains utilizing agro-waste

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Two *Streptomyces* sp., CS628 and CSWu-1, recently isolated from Korean soil were identified according to various characteristics including 16S rDNA and rRNA sequencing, respectively. Both extracellular xylanases, Xyn628 and XynWu-1, were grown on various medium and purified using different chromatographic techniques and biochemically characterized. Xyn628 was purified by single step gel permeation chromatography using Sepharose CL-6B and XynWu-1 was purified by gel permeation chromatography using sephadex G-50 followed by weak anion exchange, DEAE-sepharose fast flow, chromatography. Activity recovery of Xyn628 and XynWu-1 were 33.78 % and 16 %, respectively, whereas purity fold were 5.16 and 4.87, respectively. The molecular masses of both xylanase were approximately 18.1 kDa (Xyn628) and 37 kDa (XynWu-1) as estimated by SDS-PAGE and xylan-zymography. Resulting, molecular weight of Xyn628 is the lowest among the so far reported *Streptomyces* xylanase. N-terminal amino acid sequences of Xyn628 and XynWu-1 were AYIKEVVSRAYM, AINVLVAAL, respectively. Both the sequences are significantly different from the reported xylanase. The xylanases showed high activity and stability in extremely alkaline conditions.

Optimum temperature and pH of Xyn628 and XynWu-1 were 60 °C and 11.0, respectively. Both the xylanases were stable in a wide pH ranging between pH 5.0 ~ 13.0 after 12 h of incubation at 4 °C. Xyn628 and XynWu-1 were stable up to 65 °C and 55 °C after 1 h incubation, respectively. XynWu-1 activity was remarkably enhanced by various metal ions, detergents and organic solvents. Especially, enhanced xylanase activity of XynWu-1 at higher molar concentration by Mn^{2+} ion suggested unique among *Streptomyces*, which might be a metalloprotein and metal ions that positively stimulated its activity may be employed as catalysts in trace amounts during application The xylanases, Xyn628 and XynWu-1, were most active on beechwood xylan with K_m values of 3.1 and 1.7 mg/mL, respectively, and V_{max} of 313 and 742 mmoL/min mg, respectively. This kinetic parameter therefore importantly defines the affinity of the substrate for the enzyme. Xyn628 and XynWu-1 produced xylobiose, xylotriose and xylose, xylobiose, xylotetraose as principal hydrolyzed end products from xylan, suggesting that they are endo-xylanase in nature. Importantly, scanning electron microscopy (SEM) showed Xyn628 and XynWu-1 efficiently degraded (corncobs, wheat bran etc.) agro-industrial waste materials. In summary, Due to extremely alkaline, thermostable, cellulase free, very low molecular weight, very high effectiveness with various detergents and metal ions, series of xylooligosaccharides production etc. of the purified xylanases make these enzymes attractive for various biotechnological applications, such as biobleaching in paper and pulp industries, production of xylooligosaccharides for prebiotic in food and pharmaceutical industry, potential application in biofuel and textile industries as well as waste treatment with appropriate utilization of agro-waste products.

1. INTRODUCTION

Enzymes are the catalytic cornerstones of metabolism and as such are the focus of intense worldwide research. The use of enzymes in biotechnological processes is part long and established tradition. The utilization of enzymes for waste processing is a relatively recent development and has grown out of the increasing demands, both economical and environmental. Enzymatic transformations of the wastes originated due to diverse agricultural and industrial practices has emerged as an acceptable method for the disposal or recycling of wastes.

The recycling of resources is becoming valid and viable economic activity and is increasingly mentioned as a solution to some of the most serious problems of mankind. Enormous amounts of agricultural residues are being wasted in all over the world especially in Asia. The huge amount of residual plant biomass considered as “waste” can potentially be used to produce various value added products like oligosaccharides, biofuels, animal feeds, chemicals, enzymes etc. Wheat straw, rice straw, corncobs, tobacco stalk, wheat bran etc, are rich in lignocellulose and are considered as potential feed stocks for the industrial utilization (fig. 1.1). Since, this kind of biomass are usually left to rot or burned in the field after harvesting, utilization of these materials for industrial purpose not only solve the problem of proper disposal of the wastes, but also provide an additional income to the farmers and generate employment. These lignocellulosic substrates are reported to be an excellent substrate for growth of various industrially important bacteria, fungi, enzymes and various other valuable chemicals.

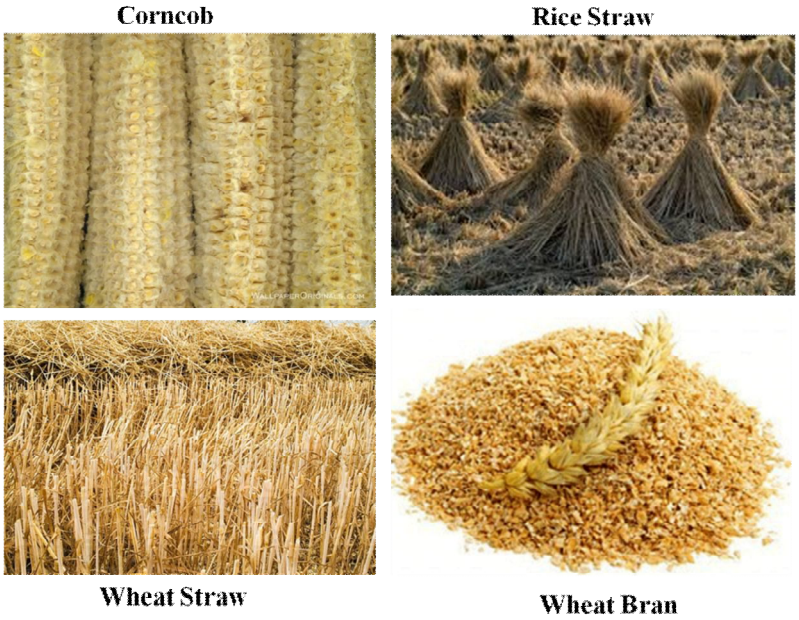

Fig. 1.1: Commonly available agro-wastes which are rich in lignocelluloses.

Importance of microbial xylanases have increased in present scenario due to its vast biotechnological applications mainly in food, animal feed, paper and pulp industries as well as for bioconversion of lignocellulosic waste into value-added products covering all the sectors of industrial enzymes market [1].

1.1 Structure and composition of lignocellulosic biomass

Lignocellulosics in the form of agriculture and forestry residues are the most abundant and inexhaustible or renewable natural resources. Lignocellulose is the major structural component of woody and non-woody plants. It mainly consists of cellulose, hemicellulose and lignin [2]. The

chemical properties of components of lignocellulosics make them a substrate of enormous biotechnological value.

Lignocellulose is the primary building block of plant cell walls. The composition of lignocellulosic biomass varied with mainly cellulose (35-50%), followed by hemicellulose (20-35%) and lignin (10-25%) [3]. The arrangement of cellulose, hemicellulose and lignin in the cell wall of lignocellulosic biomass is shown in fig. 1.2.

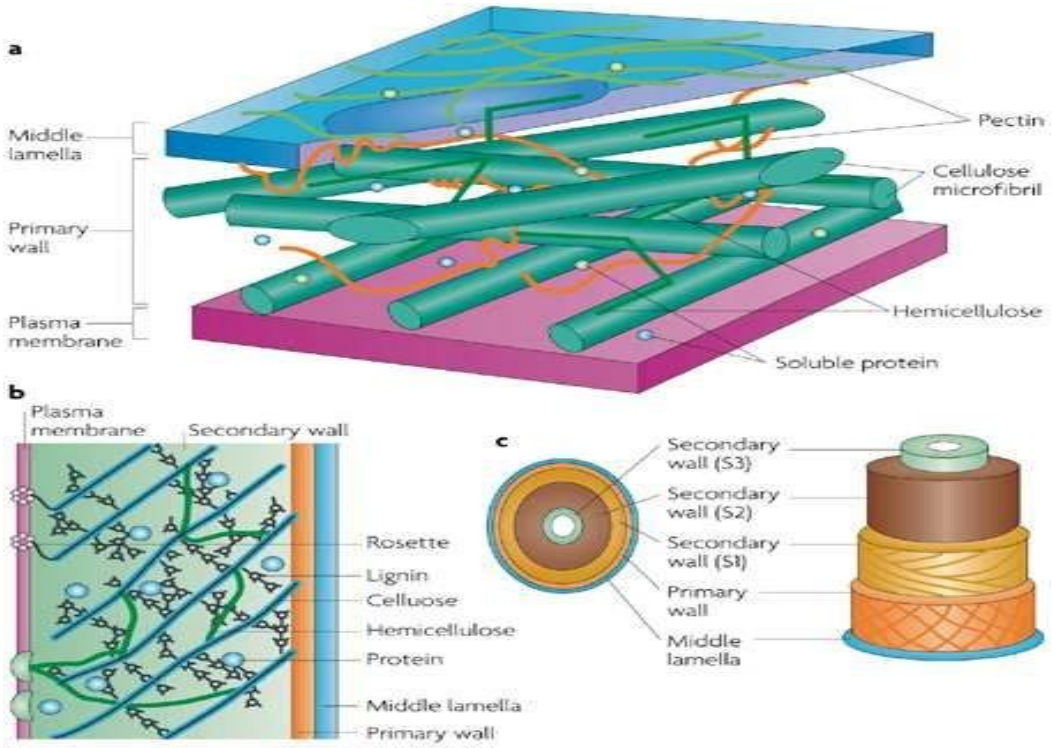

Fig. 1.2: Structure of arrangements of cellulose, hemicellulose and lignin in the cell walls of lignocellulosic biomass (**a**) cross section of cell wall, (**b**) vertical section, (**c**) schematic arrangments of cell wall in plant cells.

Cellulose fibers are embedded in a lignin polysaccharide matrix. Xylan is the main constituent of hemicellulose which plays a significant role in the structural integrity of cell walls by both covalent and non-covalent associations [2]. The cellulose is a homopolymer of glucose units linked by β-1, 4-glucosidic units, although the true repeating stereochemical unit of cellulose is cellobiose. Glucose and cellodextrins are the major end products on hydrolysis of cellulose [4].

Hemicelluloses are a heterogeneous class of polymers representing, in general, 15–35% of plant biomass and which may contain pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, and α-D-galactose) and/or uronic acids (α-D-glucuronic, α-D-4-O-methylgalacturonic and α-D-galacturonic acids). Other sugars such as α-L-rhamnose and α-L-fructose may also be present in small amounts and the hydroxyl groups of sugars can be partially substituted with acetyl groups.

Lignins are highly branched polymeric molecules consisting of phenyl-propane-based monomeric units linked together by different bonds, including alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds. It is present in the primary cell wall, imparting structural support, impermeability, and resistance against microbial attack [2]. List of certain lignocellulosic biomass with their chemical composition are mentioned in Table 1.1.

Table 1.1: Composition of lignocellulosic biomass of different agricultural residues [2].

Lignocellulosic biomass	Composition (% dry weight basis)		
	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Corn fiber	15	35	8
Corn cob	45	35	15
Corn stover	40	25	17
Rice straw	35	25	12
Wheat straw	45	28	20
Wheat bran	35	45	14
Sugarcane bagasse	40	24	25
Switch grass	45	30	12
Coastal Bermuda grass	25	35	6

1.2 Xylan: occurrence, structure and composition

Lignocellulosic biomass mainly consists of cellulose (35-50%), hemicellulose (20-35%) and lignin (10-25%) [3]. The term "hemicellulose" was first introduced by Schulze in 1891 for the fractions isolated or extracted from plant materials with dilute alkali. Hemicellulose is the second most abundant polysaccharide in nature after cellulose [2]. Xylan is the major component of hemicellulose with the high potential for degradation to useful end products. It forms covalent linkage with lignin and non-covalent linkage with cellulose and maintains structural integrity of plant cell wall [1], [5]. The main chain of xylan consists of a homo-polymeric backbone chain of endo-β-1,4-xylopyranosyl residues having side group substitution to varying degree with glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and ρ-coumaroyl groups [6]. Xylans are the main hemicellulose components of secondary cell walls constituting about 20–30% of the biomass of hardwoods and herbaceous plants. Hardwood xylans and softwood xylans are different in terms of their degree of polymerization and side group

substitution. The degree of polymerization of hard wood xylan is higher (150-200) as compared to soft wood xylan (70-130) [7], [8]. The most abundant hemicellulose in hard wood is O-acetyl-4-O-methyl glucuronoxylan. It contains on an average one α-1, 2-linked 4-O-methyl glucuronic acid substituent per 10-20 xylopyranosyl residues of main chain. About 60-70% of xylose units are esterified with acetic acid at C-2 or C-3 [9]. The presence of these acetyl groups are readily removed when xylan is subjected to alkali extraction and is responsible for partial solubility of xylan in water [7]. In soft wood plants, xylan is mainly arabino-4-O-methyl glucuronoxylan. In addition to 4-O-methyl glucuronic acid, it is also substituted by α-arabinofuranoside units [10]. Xylans from softwood are composed of arabino-4-O-methylglucuroxylans. They have a higher 4-O-methylglucuronic acid content than do hardwood xylans. The 4-O-methylglucuronic acid residues are attached to the C-2 position. Softwood xylans are not acetylated, and instead of an acetyl group they have α-L-arabinofuranose units linked by α-1, 3-glycosidic bonds at the C-3 position of the xylose. The ratio of β-D-xylopyranose, 4-O-methyl-α-D-glucuronic acid and L-arabinofuranose is 100:20:13 in softwoods [11]. Mannan-type hemicelluloses like glucomannans and galactoglucomannans are the major hemicellulosic components of the secondary wall of softwoods whereas in hardwoods they occur in minor amounts [12].

Microbial xylanases (E.C. 3.2.1.8) are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss and no side product generation [9], [13]. Enzymatic hydrolysis of xylan involves a multienzyme system including endo-xylanase (E.C. 3.2.1.8), β-xylosidase, α-L-arabinofuranosidase, acetyl xylan esterase, ferulic acid esterase and ρ-coumaric acid esterase [2]. Figure 1.3 shows the hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases.

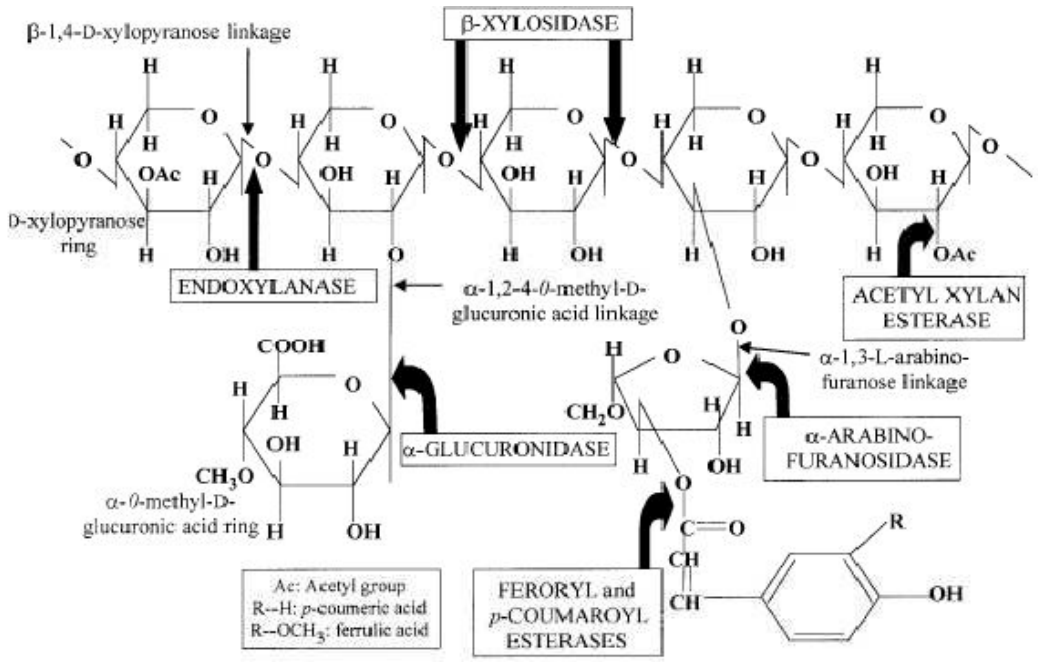

Fig. 1.3: The hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases [9].

1.3 Xylanolytic enzyme system

The complete hydrolysis of xylan requires the synergistic interaction of various enzymes because of its heterogeneous nature. The xylanolytic enzyme system mainly comprises of two types one is main chain acting enzymes and the other group forms of accessory enzymes. The main chain acting enzymes are endo-1, 4-β-D-xylanases and 1, 4-β-D-xylosidases, which hydrolyse main chain to release xylooligosaccharides and xylose. The substituent groups are cleaved by accessory enzymes such as α-L-arabinofuranosidase, α-D-glucuronidase, acetyl xylan esterases, ferulic acid esterases and *p*-coumaric acid esterases [14]. Thus, the efficient substrate hydrolysis can be achieved by the action of multiple xylanases by micro-organisms [10]. The presence of such a

multicomponent xylanolytic enzyme system is quite widespread amongst bacteria and fungi [15]. Table 1.2 shows the mode of action of each enzyme of xylanolytic enzyme system for the breakdown of xylan.

Table 1.2: List of major enzymes of xylanolytic enzyme system involved in degradation of xylan [16].

Enzymes	Mode of action
Endo-xylanase	Hydrolyzes mainly interior *β*-1,4-xylose linkages of the xylan backbone
Exo-xylanase	Hydrolyzes *β*-1,4-xylose linkages releasing xylobiose
***β*-xylosidase**	Releases xylose from xylobiose and short chain xylooligosaccharides
α-L-arabinofuranosidases	Hydrolyzes terminal non reducing α-arabinofuranose from arabinoxylan
α –glucuronidase	Releases glucuronic acid from glucuronoxylan
Acetyl xylan esterase	Hydrolyzes acetyl ester bonds in acetyl xylans
Ferulic acid esterases	Hydrolyzes feruloyl ester bonds in xylans
***ρ*-coumaric acid esterases**	Hydrolyzes *ρ*-coumaryl ester bonds in xylans

1.3.1 Endoxylanase (β-1, 4-D-xylanohydrolase, E.C. 3.2.1.8)

Endoxylanases are the hemicellulases that have been well characterized. These enzymes depolymerise xylan to short chain xylooligosaccharides of varying chain length [17]. Endoxylanases are principally of two types, non-debranching and branching [18]. The non-debranching are the non-arabinose liberating endoxylanases which fall into two categories, one

releasing xylose and xylobiose as end product and other releasing xylooligosaccharides as end products.

The former cannot act on L-arabinofuranosyl initiated branch points at β (1-4) linkages and produce only xylobiose and xylose as the major end products. The latter cannot cleave branch points at α (1-2) and α (1-3) and produce mainly xylooligosaccharides larger than xylobiose. These endoxylanases have no action on xylotriose and xylobiose. The debranching or arabinose liberating endoxylanases can be categorized in one group, that can hydrolyze branch points and produce xylooligosaccharides and arabinose as end products and the other group that cleaves the xylan and branch points releasing mainly xylobiose, xylose and arabinose. The occurrence of debranching activities in xylanases appears to be variable.

1.4 Microorganisms and production of xylanase

Production of industrial enzymes has been carried out extensively using microbial isolates, which exhibited higher productivity compared to the plants and animal sources. Thus, all living things, particularly animals, plants and microorganisms are sources of enzymes. However, for commercial applications of industrial enzymes, microorganisms are the most important source of various enzymes [19]. Xylanases have been isolated from a number of micro-organisms such as bacteria, fungi, actinomycetes, yeast, as well as plants, animals etc. however microorganisms are the major source of such enzymes. Xylanases have been reported from bacteria and fungi like *Bacillus, Streptomyces, Clostridium, Thermotoga, Thermomyces, Aspergillus, Penicillium* spp. etc. [20]. Amongst all of the xylanolytic enzymes, endo-xylanases are the best characterized and the most widely studied enzymes [17]. A number of microorganisms are reported for the production of xylanase using agro-residues. One of the big genera of Streptomycetaceae family has been

found to strong xylanase producer. Hence, production of xylanases have been reported in different *Streptomyces* species such as *Streptomyces* rameus L2001 [21], *Streptomyces* cyaneus SN32 [22], *Streptomyces chartreusis* L1105 [23], *Streptomyces* sp. SWU10 [24], *Streptomyces* sp. CS802 [25], *Streptomyces matensis* [26], *Streptomyces* sp. S38 [27], etc. If xylanases are used in pretreatment of kraft cooked pulp only the low molecular weight xylanases could successively create pores and enter the interior for better hydrolysis [28-29]. Furthermore, low molecular weight, active in alkaline pH, and cellulase free xylanases are highly desirable for biobleaching in paper and pulp industries [30]. The paper and pulp industry needs low molecular weight xylanases since the reprecipitated and relocated xylan layer over fibers require higher amount of oxidizing agents. The problem faced by these industries, however, is the cost and availability of the enzyme. Large scale cultivation of microorganisms for enzyme production remains an economical challenge. The cost of enzyme is one of the main factors determining the economics of any process. Xylanases are produced by either of the technique depending upon the culture conditions of microorganism, yield, strain and end-use of the enzymes. There are mainly two possibilities for cultivation of microbial enzymes producing strains, submerged fermentation and solid state fermentation [31].Therefore, developing better microbial strains, efficient fermentation and recovery systems as well as production from agro-wastes will always be a welcome approach in this direction.

1.5 Purification of xylanase

Purification of an enzyme is of prime importance in learning about its structural and functional properties and to predict its applications. The final degree of purity of particular enzyme depends upon its end use. The major aim behind deciding the strategy for purification is to obtain

maximum possible yield of the desired enzyme with highest catalytic activity and maximum possible purity. Most of the purification methods which are used in laboratory research can be scaled to industrial processes. Such methods are filtration, centrifugation, ultrafiltration, diafiltration, precipitation and chromatography such as ion-exchange chromatography and gel filtration chromatography. Xylanases are considered to be mostly extracellular but intracellular xylanases have also been reported in some organisms such as *Ruminicoccus flavefaciens* [32], *Bacillus stearothermophilus* T-6 [33], etc.

Purification of xylanases to homogeneity is necessary for detailed biochemical and molecular studies and for the successful determination of their primary amino-acid sequences and their three dimensional structures [34]. The key for successful and efficient purification strategies is the selection of appropriate techniques that maximize yield and purity with minimum number of steps [35]. Microbial xylanases are mainly purified by chromatographic methods, using from two to five purification steps and providing recovery yields ranging from 0.2 to 78%. Ion exchange and gel permeation chromatography or the combinations of both are the most common methods. Both cation and anion exchange chromatography are used for the purification of xylanases. The use of matrices in the ion exchange chromatography differs from type to type of enzyme and microorganisms. As expected, lower yields were generally obtained when a greater number of purification steps are used. Less number of steps generally has higher recovery yields. Preparation and purification strategy used in this study are shown in figure 1.4.

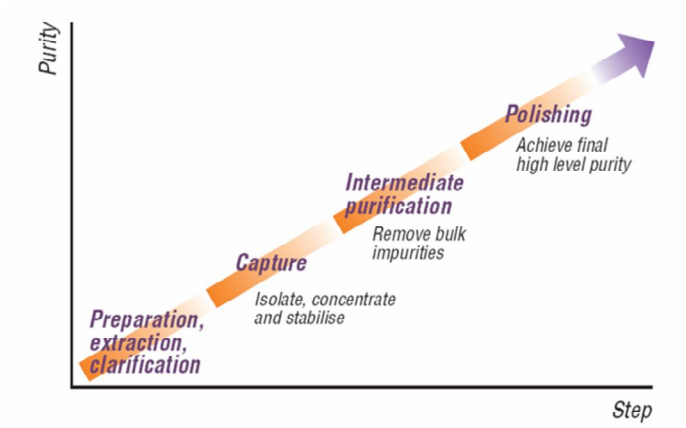

Fig. 1.4: Schematic presentation of preparation and purification strategy used in this study.

1.6 Applications of xylanases

Importance of microbial xylanases have increased in present scenario due to its immense biotechnological applications in mainly food, animal feed, paper and pulp industries as well as for bioconversion of lignocellulosic waste into value-added products covering all the sectors of industrial enzymes market [1]. Xylanases have also shown an immense potential for increasing the production of several useful products in a most economical way. The recent application of xylanase is in production of xylooligosaccharides which have wide use as prebiotic and food additive in food industry. Xylooligosaccharides (XOS) are sugar oligomers made up of xylose units with the chain length of 2 to 10 and are considered as non digestible food ingredients [36]. Lignocellulosic biomass such as wheat bran, wheat straw, rice straw, corncobs, tobacco stalk, sunflower stalk etc. are widely used for production of XOS. Utilization of such biomass as raw materials offers economic and ecological benefits, since it is biorenewable, widely distributed and

abundant resources. A list of recently reported applications of xylanases in various sectors is shown in Table 1.3.

Table 1.3: Compilation of recently reported applications of xylanases in various sectors [37].

Sectors	Applications
Xylooligosaccharides Production	Prebiotics, reduce cholesterol level, increase Ca^{+2} availability, antioxidant activity, antimicrobial, antiallergy, anti-infection, anti-inflammatory properties, selective cytotoxic activity, immunomodulatory action, cosmetics and pharmaceuticals, etc.
Bioconversion of lignocellulosic materials	Fuel ethanol, bioethanol, etc.
Paper and pulp industry	Prebleaching of kraft pulp, etc.
Animal feed	Improving both the weight gain of chicks and their feed conversion efficiency.
Food industry	Improving the quality of bread, i.e. better flavour, taste, softness and overall acceptability. Clarifying must and juices, liquefying fruits and vegetables, improve the digestibility of ruminant feeds, thickening agents, or as fat substitutes and as antifreeze food additives.
Textile industry	Retting of flax, jute, ramie, hemp, etc.
Others	Food processing for aromatizing musts, wines, and fruit juices. Improve cell wall maceration for the production of plant protoplasts.

1.7 Problem delineated

Most of the Asian countries are dependent on agricultural. Huge quantities of plant biomass are produced during agricultural processes. Among all of them, very less amount of this plant biomass is used for animal feed and miscellaneous uses while rest is generally considered as "waste" and is usually left to rot or burned in the field after harvesting. However, these so called "wastes" are richest sources of lignocellulose and can be potentially used to produce various value added products such as biofuels, chemicals, enzymes etc. Apart from this solid waste, large amount of waste waters are generated from alcohol industries. The waste released by these distilleries is considered to be high strength and as such cannot be disposed in the environment. On the other hand, distillery spent wash also contains significant quantity of valuable nutrients which can support the growth of microorganisms and plants. There is an increased surge to develop a sustainable technology for the utilization of these wastes. Production of microbial xylanases and their application in eco-friendly production of fermentable sugars for bioethanol and xylooligosaccharides, a value added food ingredient using agro-residues are important areas. Reductions in the cost of xylanases as well as these valuable end products are important issues for industries. Thus, the present study was focused on utilization of agricultural residues for the production of industrially important enzyme, xylanase and in turn application of xylanase for production of value added products utilizing agro-residues which may serve the better utilization of waste and solve the problem for their disposal.

1.8 Objectives of the present study

In the context of the above discussion, the present study was aimed with the following objectives:

- ✓ Screening of potential xylanase producers from *Streptomyces* species.
- ✓ Production of xylanase using commercial xylan and agro-residues.
- ✓ Purification of endo-xylanase and its characterization.
- ✓ Biotechnological applications of xylanase.

2. MATERIALS & METHODS

2.1 Materials

Xylose, beechwood and birchwood xylan were purchased from Sigma-Aldrich (St Louis, MO, USA). Sepharose CL-6B was purchased from Amersham Bio-Sciences AB (Uppsala, Sweden). Sephadex G-50 was purchased from Pharmacia BioProcess (Uppsala, Sweden). DEAE Sepharose (fast flow) was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Thin layer chromatography silica gel plates were from Merck (Darmstadt, Germany). Xylobiose, Xylotriose and Xylotetrose were from Megazyme (Ireland). All other chemicals used were of analytical grade.

2.2 Strains Isolation and Identifications

Strain CS628 was isolated from Jeonnam province South Korea and identified as a *Streptomyces* species according to various characteristics including 16S rDNA sequencing, according to our previous report [38]. On the other hand, strain CSWu-1 was isolated from Korean soil and identified as a *Streptomyces* species according to various characteristics including 16S rRNA sequencing, according to our previous report [39].

2.3 Production of Xylanases

2.3.1 Production of Xyn628

Strain CS628 was identified as a strong xylanase producer using xylan-agar plate [40]. For the production of xylanase (Xyn628), the strain was selected and cultivated into the xylan rich agro-waste, wheat bran, medium as a sole carbon source with the following composition containing 2.0 % wheat bran, 0.5 % yeast extract, 1 % tryptone, 0.75 % KH_2PO_4, 0.15 % K_2HPO_4 and

0.05 % $MgSO_4$. Cultivation was carried out in 1 L Erlenmeyer flasks containing 250 mL medium in a shaker set at 30 °C and 120 rpm for 4 days.

2.3.2 Production of XynWu-1

Strain CSWu-1 was identified as a strong xylanase producer using xylan-agar plate [40]. For the production of xylanase, *Streptomyces* sp. CSWu-1 was cultivated in 250 mL Erlenmeyer flasks containing 50 mL of optimized production medium supplemented with 1.25 % beechwood xylan, 0.5 % yeast extract, 1 % tryptone, 0.75 % KH_2PO_4, 0.15 % K_2HPO_4 and 0.05 % $MgSO_4$; under shaking conditions (120 rpm) at 28 °C for 7 days. Furthermore, the production of xylanase was also evaluated by using agro-waste materials like corn cob and wheat bran. For both cases, under same conditions commercial xylan was replaced by corn cob and wheat bran and production were performed. Corncob and wheat bran were bought from local Nam-Gwangju market; grains were removed, washed, dried, and then grounded for further use.

2.4 Determination of Enzyme activity

Xylanase activity of the enzymes was assayed according to Miller [41]. The reaction mixture contained 0.1 mL of 0.5 % (w/v) beechwood xylan and 0.1 mL of suitably diluted enzyme solution (50 mM, KCl/NaOH buffer, pH 11.0) at 60 °C for 5 min (Xyn628) and 10 min (XynWu-1), respectively. The amount of reducing sugar liberated was determined by the dinitrosalicylic acid (DNS) method using xylose as a standard. One unit of xylanase activity was defined as the amount of enzyme that catalyzes the release of 1 μM of xylose equivalent in one minute under the standard assay conditions. Cellulase activity was evaluated using sigma cell cellulose, carboxymethyl cellulose, avicel and an artificial substrate such as, paranitrophenyl D- celobioside (pNPC) and paranitrophenyl-β- D glycopyranoside (pNPG).

2.5 Protein determination

The protein concentration was determined according to the Bradford method [42] using bovine serum albumin as a standard.

2.6 Purification of enzymes

2.6.1 Purification of Xyn628

The crude extracellular xylanase was obtained by centrifuging the culture broth at 6,000 × *g* for 1hour (h) and 30 min at 4 °C. The crude supernatant was subjected to 30–75 % ammonium sulfate precipitation. Proteins were recovered by centrifugation at 6,000 × g for 1 h and 30 min, dialyzed against 10 mM Tris/HCl (7.0) and concentrated with an ultra filtration membrane (YM 5 kDa, Millipore Corp, Amicon Danvers, MA, USA). Afterwards, the dialyzed enzyme solution was applied to Sepharose CL-6B column (1.7 cm × 77.5 cm). The column was pre-equilibrated with 10 mM Tris/HCl buffer, pH 7.0, and then washed with the same buffer at a flow rate of 5.29 cm/h (2.5 mL in each fraction). This purification step yielded one active xylanase fraction, and its homogeneity was checked by SDS-polyacrylamide gel electrophoresis. Absorbance at 595 nm was used to monitor the protein in purification steps.

2.6.2 Purification of XynWu-1

The crude extracellular xylanase was obtained by centrifuging the culture broth at 6,000 × *g* for 45 min at 4 °C. The crude supernatant was subjected to 30–75 % ammonium sulfate precipitation. Proteins were recovered by centrifugation at 6,000 × g for 1 h, dialyzed against 10 mM Tris/HCl (7.5) and concentrated with an ultra filtration membrane (YM 30 kDa, Millipore Corp, Amicon Danvers, MA, USA). Afterwards, the dialyzed enzyme solution was applied to Sephadex G-50 column (2.3 cm × 76.5 cm). The column was pre-equilibrated with 10 mM Tris/HCl, pH 7.5, and

then washed with the same buffer at a flow rate of 2.17 cm/h (3 mL in each fraction). The xylanase from Sephadex G-50 was further purified by DEAE-sepharose fast flow column (1.2 cm × 11 cm) pre-equilibrated with 10 mM Tris–HCl, pH 7.0. The bound proteins were eluted with 0–1.0 M KCl gradient at a flow rate of 1 mL/min. This purification step yielded active xylanase fraction, and its homogeneity was checked by SDS-polyacrylamide gel electrophoresis. Absorbance at 595 nm was used to monitor the protein in purification steps.

2.7 Determination of enzyme purity

The purified enzymes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % (w/v) polyacrylamide slab gel according to [43]. For reference proteins, protein size marker (MBI, Fermentas) was used. Proteins were observed by staining with Coomassie Brilliant Blue R-250 and then destaining with a solution containing methanol: glacial acetic acid: distilled water = (1:1:8 by vol). Molecular weight was estimated by comparing with the relative mobility of the reference protein. In addition, xylan zymography was performed as described in [44].

2.8 Effect of pH on activity and stability of the xylanases

The effect of pH on the activity of xylanases (Xyn628 and XynWu-1) were carried out at 60 °C at various pH values (3.0–13.6) using 10 mM pH buffers, that was; Citric Acid/Sodium Phosphate (pH 3.0–7.5), Tris/HCl (pH 7.0–9.5), CAPS (pH 9.0–11.0), and KCl/NaOH (pH 11.0–13.6). To determine the pH stability of the enzyme, the xylanases were incubated in appropriate buffers of different pH (as mentioned above) at 0-4 °C for 12 h, respectively, and the residual xylanase activities were measured under the standard assay conditions.

2.9 Effect of temperature on activity and stability of the xylanases

The optimum temperature for xylanase activity was determined by incubating the enzymes (Xyn628 and XynWu-1) at different temperatures (40–80 °C) in 10 mM KCl/NaOH (pH 11.0). To determine the temperature stability of the enzyme, the xylanases (Xyn628 and XynWu-1) were incubated at different temperatures (20–80 °C) at the above optimum conditions for 1 h. After incubation, the residual xylanase activity was measured by the standard assay conditions.

2.10 Effect of detergents

The effect of different types of detergents on Xyn628 and XynWu-1 activity were performed by adding at a concentration of 0.25 % and 1 mM respectively. Enzymes activity of detergents were investigated by adding Triton X-100, Tween-20, Tween-80, polyoxyethylene-4-laurylether, deoxycholic acid (DCA), SDS and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). Enzymes activity in absence of any additives were measured and considered as 100 % under the standard assay conditions.

2.11 Effect of metal ions, and other additives

The effects of various metal ions, at a concentration of 1 mM (Xyn628), 1 mM and 5 mM (XynWu-1), on xylanase activity were investigated by adding monovalent (Na^+ and K^+) and divalent (Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+} and Fe^{2+}) metal ions to the reaction mixture, respectively. In addition, effect of Mn^{2+} ions on XynWu-1 activity was investigated up to 12 mM. Conversely, the effects of oxidizing and reducing agents on XynWu-1 activity were tested by adding hydrogen peroxide, sodium perborate, β-mercaptoethanol and dithiothretol at a concentration of 5 mM. On the other hand, the effect of chelating agents on XynWu-1 activity was evaluated with ethylenediamine tetra-acetic acid (EDTA) and ethylene glycol tetra-acetic acid

(EGTA). In all the circumstances, the enzyme activity without any additives was measured and considered as 100 % under the standard assay conditions.

2.12 N- terminal amino acid sequences

The N-terminal amino acid sequence of Xyn628 and XynWu-1 were determined by Edman degradation using a Procise Model 492 protein sequencer (Applied Biosystems, CA, USA).

2.13 Substrate specificity of xylanases

To evaluate the substrate specificity of the purified enzymes, the enzymes (Xyn628 and XynWu-1) were incubated with 0.5 % (w/v) of each substrate and the activity was measured under standard assay conditions of each enzymes.

2.14 Kinetic parameter of xylanases

A hyperbolic computer software program was used for the determination of kinetic parameter. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined by Eadie-Hofstee plot. In experiment, different concentrations (2.5-40 mg/mL) of beechwood xylan, and constant enzyme concentrations, 0.18 µg (Xyn628) and 0.09 µg (XynWu-1), were prepared and assays were done under standard assay conditions at optimal condition of each enzymes.

2.15 Mode of hydrolysis and potential application of xylanases in xylooligosaccharides production

To determine the mechanism of action of xylanases and potential application in the production of xylooligosaccharides, a simple qualitative but substantiative evaluation method of thin layer chromatography (TLC) was applied according to [44] with minor modification. Suitably diluted purified xylanases were incubated with 0.5 %, Xyn628, and 1 %, XynWu-1, (w/v) beechwood xylan in 50 mM KCl/NaOH (pH 11.5) at 50 °C (Xyn628) and 40 °C (XynWu-1). The aliquots were taken out at different time intervals and analyzed by thin-layer chromatography (TLC). The plates (6 cm × 6.5 cm) were developed with chloroform: acetic acid: water (6: 10: 2, v/v) followed by heating for few minutes at 130 °C in a heating plate after spraying the plates with a methanol: sulfuric acid mixture (95: 5, v/v). A mixture of xylooligosaccharides consisting of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) (10 mg/mL) were used as the standard. Further, evaluation of degradation of corn-cob powder and wheat bran pulp by Xyn628 and XynWu-1 were analyzed through scanning electron micrograph according to [21]. For the experiment, corncob and wheat bran were treated with 20 U/g and 2000 U/g of Xyn628 and XynWu-1 at optimum pH and 50 °C for 2 h, whereas enzyme sample replaced by buffer was taken as control sample

3. RESULTS AND DISCUSSION

3.1 Strains isolation and identification

In the present study, a bacterial strain CS628 was isolated from Korean soil. According to our previous report [38], the strain CS628 already published as a potent Phospholipase D from *Streptomyces* sp. CS628. Furthermore, 16S rDNA sequence of the strain showed 100 % homology with *Streptomyces cyanecfuscatus* (Accession no. AY999770) and *S. cavourensis* subsp.*washingtonesis* NRRL B-8030T (Accession no. DQ026671). The results showed that it was a *Streptomyces* strain and therefore we designated it as *Streptomyces* sp. CS628. On the other hand, a bacterial strain CSWu-1 was isolated from Korean soil. Based on 16S rRNA sequences (Fig. 2.1) and other characteristics the strain was identified as *Streptomyces* sp. CSWu-1 according to our previous report [45]. A phylogenetic tree based on 16S rRNA is presented in fig. 2.2.

GGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAT
GAACCTCCTTCGGGAGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGG
CAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCG
GATACCACTCTTGCAGGCATCTGTGAGGGTTGAAAGCTCCGGCGGTGAAG
GATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGG
CGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT
TGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAA
GAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCA
AGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCAC

GTCGGGTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGG

CTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAA

TGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCA

TTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC

CTGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCGACATTCCA

CGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACG

GCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCG

GAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGA

CATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACA

GGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC

CCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGT

GATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGA

CGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACA

ATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAA

AGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGG

AGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGG

CCTTGTACACACCGCCCGTCACGTCACGAAAGTTGGTAACACCCGAAGCC

GGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTAGCGAT

Fig. 2.1: Full 16S rRNA sequences of strain CSWu-1

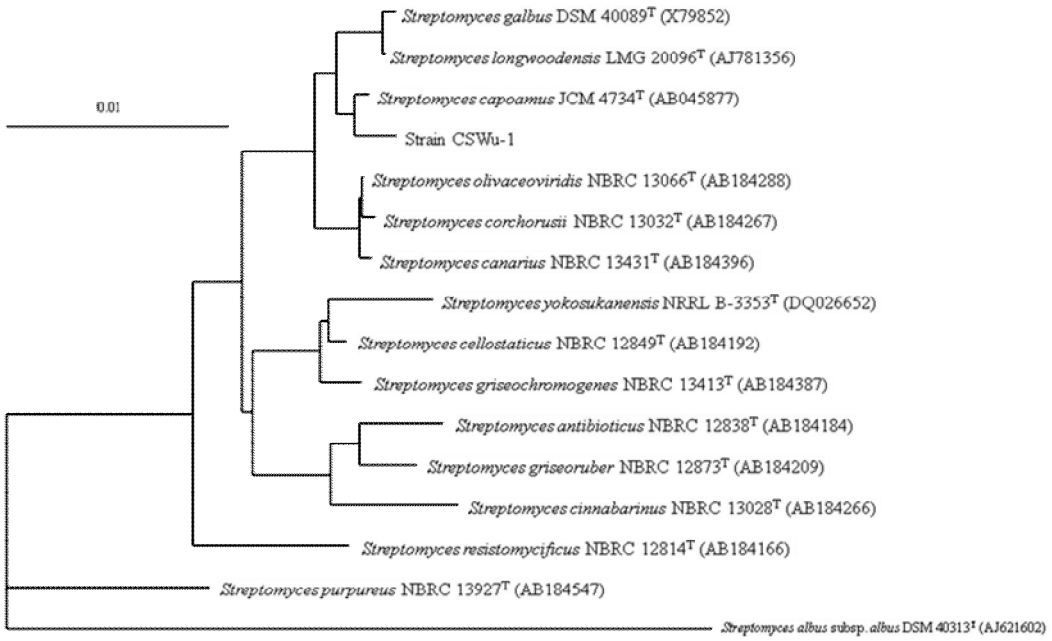

Fig. 2.2: Phylogenetic tree of strain CSWu-1; Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain CSWu-1 and representative members of the genus *Streptomyces*. *Streptomyces albus* subsp. *albus* DSM 40313^T (AJ621602) was used as an out-group. The bar represents 0.01 nucleotide substitutions per site.

3.2 Production of enzymes

3.2.1 Production of Xyn628

For the production of enzyme, CS628 strain was cultivated in the xylan rich agro-waste, wheat bran, medium as a main carbon source as mentioned in the "Materials and Methods" section. The xylanase was produced and harvested after 4 day where it showed maximum activity (fig 2.3).

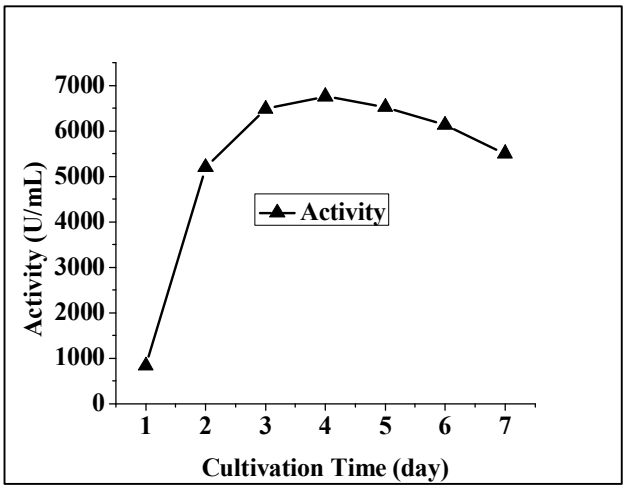

Fig. 2.3: CS628 strain was grown on optimized liquid medium containing 2 % wheat bran as a sole carbon source.

Production of xylanase using wheat bran powder as a substrate produced (6757 U/mL) 9.38 -fold higher activity than that of xylanase from *S. cyaneus* SN32 [21] using wheat bran. However, the highest xylanase activity obtained in *S. olivaceoviridis* E-86 [46] and *S. rameus* L2001 [21] were 1385 U/mL and 1810.9 U/mL using corncob, respectively. Wheat bran, wheat straw, rice straw, corncobs, tobacco stalk, etc. are rich in lignocelluloses and considered as potential feed stocks for the industrial utilization. Utilization of these materials for industrial purpose not only solves the proper disposal of the wastes, but also provides an additional income to the farmers and generates employment. Thus, it is observed that, the strain CS628 is a potent xylanase producer utilizing agro-wastes, wheat bran, as a substrate which might be applied in various bio-industries considering cheap, readily available, and unnecessarily occupying xylan rich agro-residues.

3.2.2 Production of XynWu-1

For the production of XynWu-1, CSWu-1 strain was cultivated in the xylan medium as mentioned in the "Materials and Methods" section. The xylanase was produced and harvested after 4 day where it showed maximum activity (fig. 2.4).

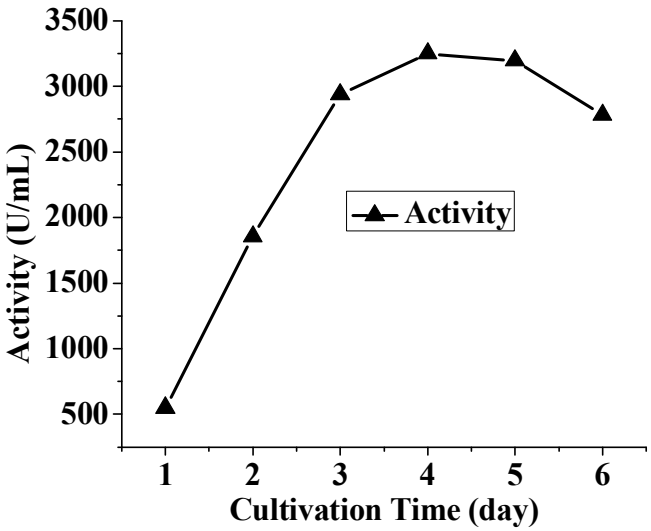

Fig. 2.4: CSWu-1 strain was grown on optimized liquid medium containing 1.25 % beechwood xylan as a sole carbon source.

Production of xylanase using corncob as a substrate produced (6061 U/mL) 1.8 fold higher activity than that of commercial beechwood xylan (3253 U/mL) as well as higher than from our recent report [25]. On the other hand, production of xylanase using wheat bran as a substrate produced (3252 U/mL) which is almost similar to commercial beechwood xylan.

Even though, the highest xylanase activity obtained in *S. rameus* L2001 [21] and *S. olivaceoviridis* E-86 [46] were 1385 U/mL and 1810.9 U/mL using corn cob and wheat bran, respectively. In recent trends, production of microbial enzymes, especially lignocellulosic biomass, by utilizing agro-waste is a great interest in the research field because it is readily available, cheap, and renewable and keeps our environment free of pollution. Thus, it is observed that, the strain CSWu-1 is a potent xylanase producer using agro-waste corn cob, wheat bran and others as a substrate which could be applied in various bio-industries considering low economic cost and unnecessarily occupying xylan rich agro-residues.

3.3 Purification of enzymes

3.3.1 Purification of Xyn628

A single step Gel filtration using Sepharose CL-6B of the ammonium sulfate fraction (30-75 %) yielded one active xylanase fraction and its elution profile was illustrated in figure 2.5.

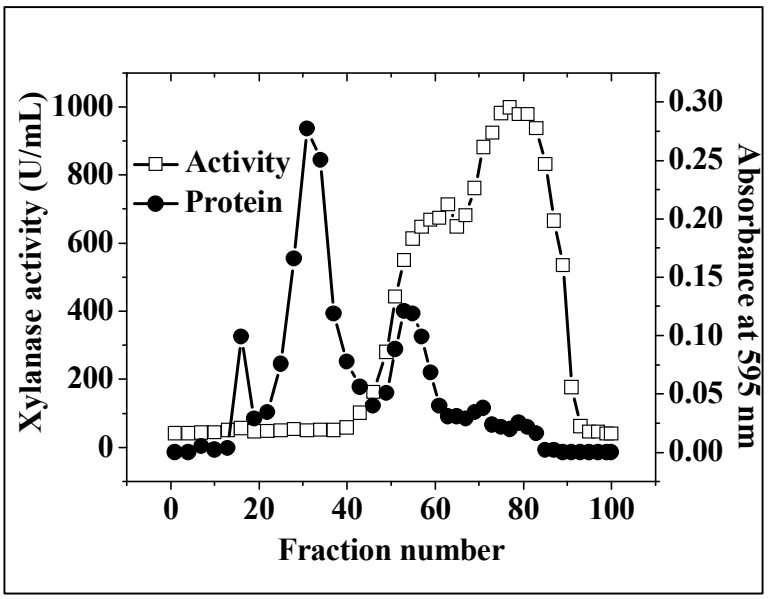

Figure 2.5: Elution Profile of Xyn628 after gel filtration chromatography using Sepharose CL-6B column (1.7 cm × 77.5 cm).

A summary of the Xyn628 purification steps from the cell-free culture supernatant CS628 was given in Table 2.1.

Table 2.1: Purification summary of Xyn628

Purification Step	Total protein (mg)	Total Activity (U)	Specific activity (U/mg)	Yield (%)	Purity fold
$(NH_4)_2SO_4$ Fractionation	2.29	459836	200802	100.0	1.0
Sepharose CL-6B	0.15	155324	1035493	33.78	5.16

At the end of chromatography, a 5.16-fold purity and 33.78 % activity was recovered. The purity fold was higher than the Xylanase from *S. cyaneus* SN32 [22], and *S.* sp. CS802 [25] where as the activity recovery was almost similar with *S. chartreusis* L1105 [23].

Analysis of the purified xylanase on sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) and activity stained gel electrophoresis (zymogram) were shown in (Fig. 2.6).

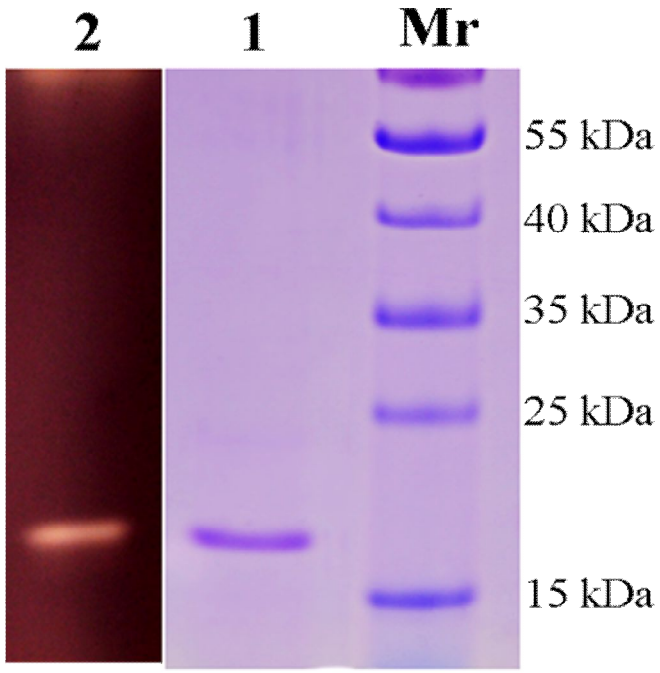

Fig. 2.6: SDS-PAGE & Zymography of purified xylanase from *Streptomyces* sp. CS628. Lanes Mr, protein molecular weight marker, lane 1, purified xylanase after Sepharose CL-6B on SDS-PAGE and lane 2, purified band on zymography.

The enzyme migrated as a single band with a molecular weight of approximately 18.1 kDa confirming its homogeneity. The xylanase showed a relatively clear band on the zymogram gel, detected by congo red staining, indicating that it was active. According to SDS-PAGE and Zymography, the xylanase showed smallest molecular weight among purified *Streptomyces* so far reported to our best knowledge. However, the xylanases from *S. cyaneus* SN32 [22] *S. rameus* L2001 [21] and *S. matensis* DW67 [26] are closer to the molecular mass of Xyn628. Furthermore, it is notable to mention that the molecular mass of Xyn628 is the smallest among so far reported purified Streptomyces strains as illustrated in Table 2.3.

3.3.2 Purification of XynWu-1

Purification of XynWu-1 was performed by gel filtration using Sephadex G-50 of the ammonium sulfate fraction (30-75 %) yielded a single major xylanase peak (Fig. 2.7a).The active fractions were pooled, concentrated and purified using DEAE-sepharose (fast flow) which eluted two subsequent xylanolytic peaks (Fig. 2.7b), and major peak retained the pure protein.

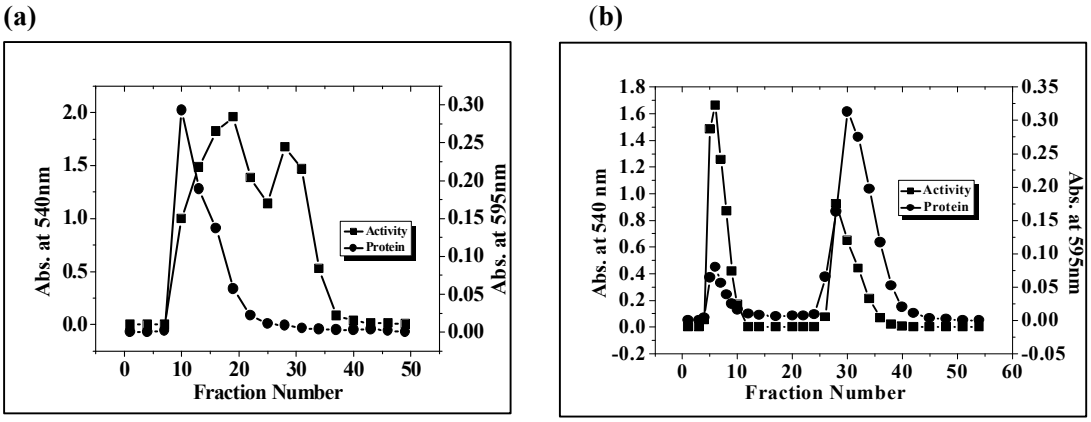

Figure 2.7: Elution Profile of XynWu-1 from *Streptomyces* sp. CSWu-1. (a) Gel filtration chromatography using Sephadex G-50 column (2.3 cm × 76.5 cm). The proteins were eluted at a flow rate of 2.7 cm/h. (b) Weak anion exchange chromatography using DEAE-sepharose (fast flow) column (1.2 cm × 10 cm). The proteins were eluted at a flow rate of 26.54 cm/h with a linear gradient of 0–1.0 M KCl.

A summary of the XynWu-1 purification steps from the cell-free culture supernatant CSWu-1 is given in Table 2.2.

Table 2.2: Purification summary of XynWu-1

Purification Step	Total protein (mg)	Total Activity (U)	Specific activity (U/mg)	Yield (%)	Purity fold
$(NH_4)_2SO_4$ Fractionation	4.41	125410	28438	100.0	1.0
Sephadex G-50	2.16	64502	29862	51.43	1.05
DEAE-sepharose FF	0.14	20063	138611	16.0	4.87

At the end of chromatography, a 4.87-fold purity and 16.0 % activity was recovered. The purity fold and activity recovery were slightly higher than the Xylanase from *S.* sp. [25-26], respectively.

Analysis of the purified xylanase on SDS-PAGE and activity stained gel electrophoresis (zymogram) are shown in (Fig. 2.8).

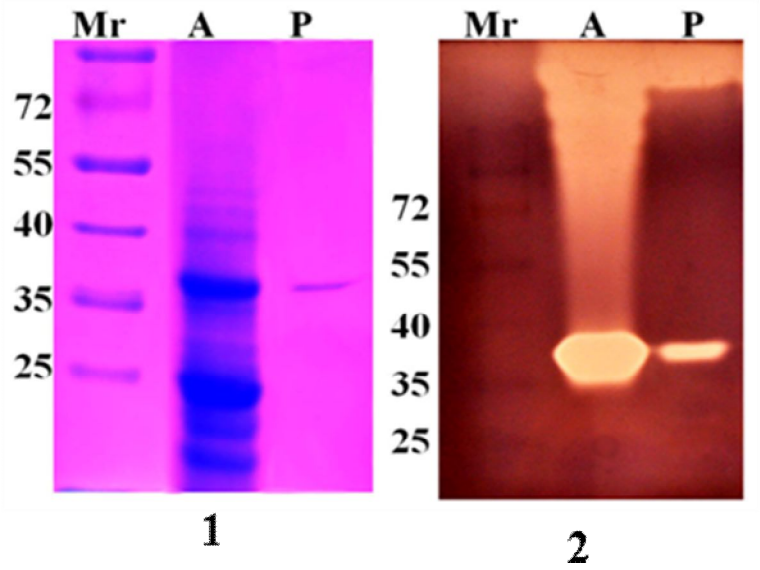

Fig. 2.8: (1) SDS-PAGE and; **(2)** Zymography of purified xylanase from *Streptomyces* sp. CSWu-1. Lanes Mr, protein molecular weight marker (kDa), lane A, ammonium sulphate fraction on SDS-PAGE and zymography, lane P, purified xylanase after DEAE-sepharose FF on SDS-PAGE and zymography.

The enzyme migrated as a single band with a molecular mass of approximately 37 kDa confirming its homogeneity. The xylanase showed a relatively clear band on the zymogram gel, detected by congo red staining, indicating that it was active. Its molecular mass is able to compared with *S. thermocyaneociolaceus* [47], *S.* sp. 7b [48] and shown in Table 2.3.

3.4 Effect of pH on activity and stability of the xylanases

Enzyme activity is remarkably affected by pH. The effect of pH on the xylanase was determined within the pH range of 3.0 to 13.0. The influence of pH in the Xyn628 and XynWu-1 activity and

stability are reported and compared with other related xylanases from *Streptomyces* are presented in Table 2.3.

Table 2.3: Comparative study of CS628 and CSWu-1 xylanase with other *Streptomyces* sp. Xylanases

Stain Name		**Optimum**		**Stability**		**Reference**
	Mol. Wt	pH	Temp	pH	Temp	
***S.* sp. CS628**	18.1	11	60	5-13	60	Present study
***S.* sp. CSWu-1**	37	11	60	7-13	50	Present study
***S. olivaceoviridis* E-86**	23	6	60	2-11	40	[49]
S. lividans	43	6	60	-	37	[50]
***S.* sp strain S38**	-	6	55-60	3-10	50	[51]
***S. cyaneus* SN32**	20.5	6	60-65	4-9.5	60	[52]
***S. matensis* DW67**	21.2	7	65	4.5-8.0	55	[26]
***Streptomyces* sp. Ab 106**	-	6	60	-	-	[53]
S. thermocyaneociolaceus	35	5	60	4.5-10.5	60	[47]
***S. rameus* L2001**	21.1	5.3	70	2.2-11.3	70	[21]
***S. megasporus* DSM 41476**	47.6	5.5	70	4-11	60	[54]
***S.*sp. 7b**	30	6	50	6-9	50	[48]
***S.* sp TN119**	35.9	7	50	2-11	37	[55]
***S.*sp. SWU10 (XynSW2A)**	31	6	60	3-9	80	[24]
***S.*sp. SWU10 (XynSW2B)**	44	6	60	2-9	60	[24]

Both the enzymes showed maximum activity at pH 11.0 whereas it was active from pH 5.0 to 13 (Xyn628) and 7.0 to 13.0 (XynWu-1), respectively. The relative activity for Xyn628 at pH 5.0 and 13.0 were 86 % and 27 % of the maximum activity, respectively (fig. 2.9).

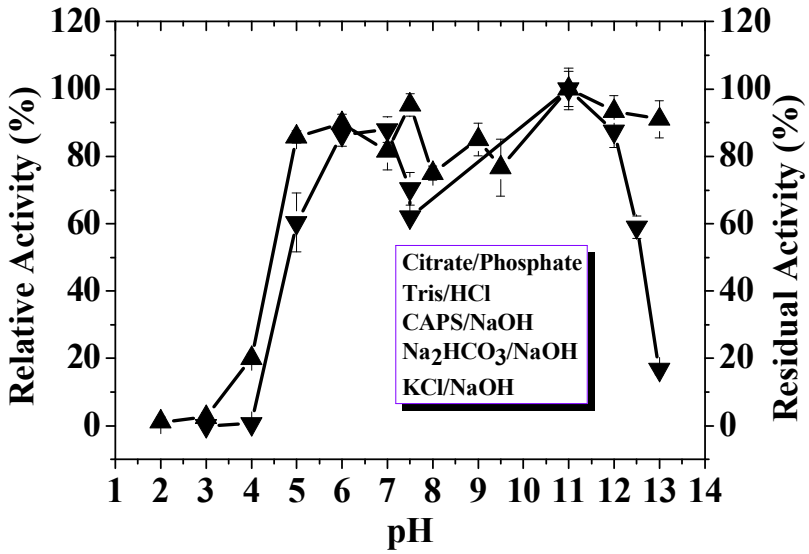

Fig. 2.9: Effect of pH on the activity (▲) and Stability (▼) of Xyn628. pH stability was determined by measuring the residual enzyme activities after incubating the enzyme at various pHs.

Besides, the relative activity for XynWu-1 at pH 7.0 and 13.0 were 86 % and 27 % of the maximum activity respectively (fig. 2.10). Moreover, Xyn628 and XynWu-1 was highly stable in alkaline pH ranged from pH 5.0, 7.0 to 13.0, respectively, whereas Xyn628 and XynWu-1 showed maximum stability at pH 11.0 and 12.0, respectively (fig. 2.9 and 2.10).

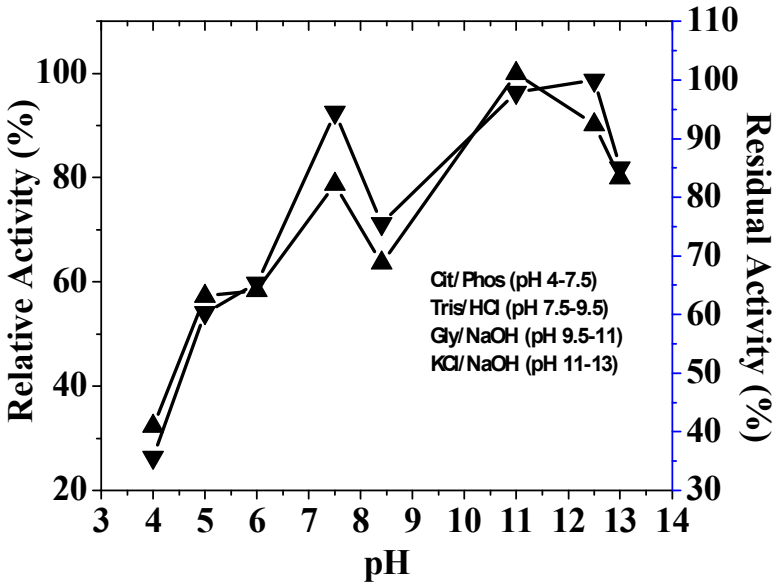

Fig. 2.10: Effect of pH on the activity (▲) and Stability (▼) of XynWu-1. pH stability was determined by measuring the residual enzyme activities after incubating the enzyme at various pHs.

Even though, the stability of these enzymes, Xyn628 and XynWu-1, were 86 % and 94 % even at pH 13.0, respectively. This unparalleled feature denotes that both the enzymes are extremely alkaline xylanase. The reasons behind such stability on broad range of pH may be due to the reversible denaturation of the protein so that there is no effect on the activity of the enzyme on incubation at different pH. Another report mentioned that amino acid(s) near the catalytic center of the enzyme are responsible for such hyperalkaline nature [39]. However, their pH are even more alkaline than *S. rameus* L2001 [21] and all other xylanases illustrated in Table 2.3. This inherent property makes Xyn628 and XynWu-1 more superior among so far reported enzymes; and hence we believe that these enzymes are novel in its kind.

Tolerance to pH is a crucial factor in the successful application of xylanases in industry. The alkaline tolerance of the xylanase isolated from *Streptomyces* sp. CS628 and CSWu-1 indicates its significant promise for application in the production of oligosaccharides as well as biobleaching in paper and pulp industry.

3.5 Effect of temperature on activity and stability of the xylanases

The optimal temperature of Xyn628 and XynWu-1 were 60 ºC at pH 11.0. At the temperatures between 50, 45 ºC and 75, 70 ºC, the enzymes, Xyn628 and XynWu-1, activity were almost equal to and greater than 70 % of the maximum activity, respectively (fig. 2.11).

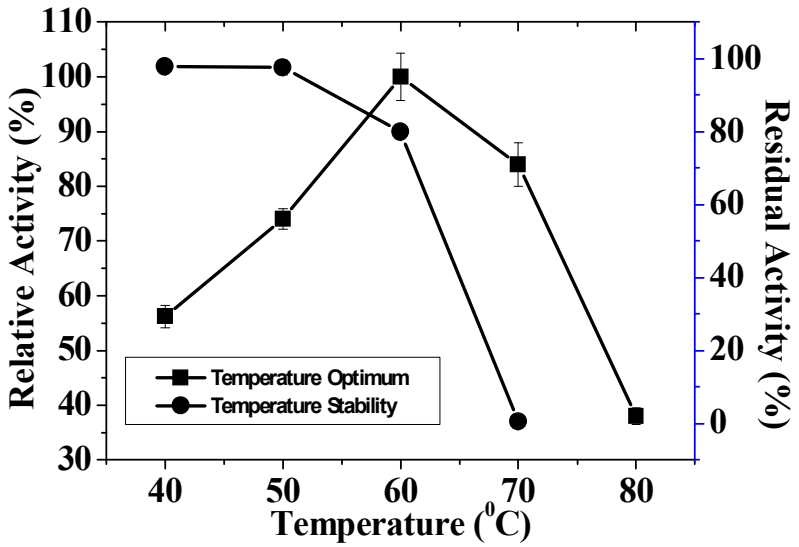

Fig. 2.11: Effect of Temperature on the activity (■) and stability (●) of Xyn628. Thermostability of the enzyme was evaluated by measuring the residual activities after incubating enzyme samples in various temperatures.

The influence of temperature on Xyn628 and XynWu-1 activity and stability are reported and compared with other related xylanases in Table 2.2. Xyn628 and XynWu-1 were completely stable after incubation at 50 and 40 °C for 1h, respectively (Fig. 2.12).

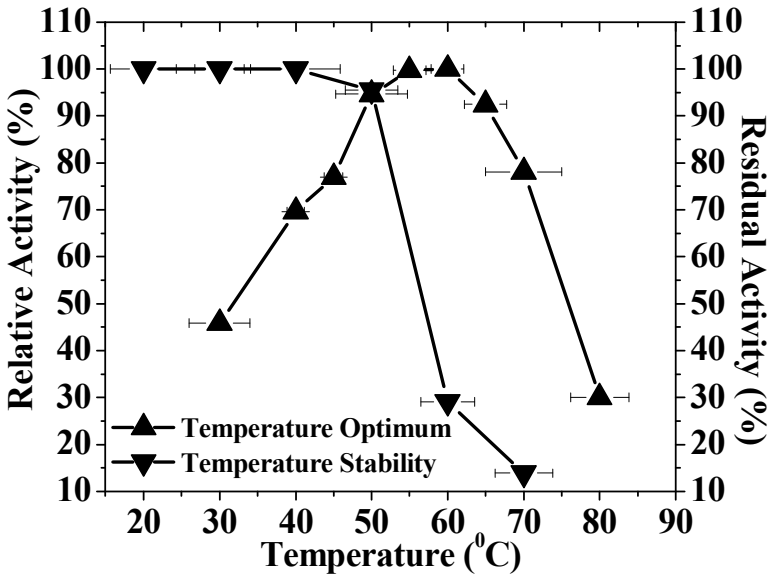

Fig. 2.12: Effect of Temperature on the activity (▲) and stability (▼) of XynWu-1. Thermostability of the enzyme was evaluated by measuring the residual activities after incubating enzyme samples in various temperatures.

Even though, almost 50 % (Xyn628) of the maximum activity was found in existence at 1 h incubation at 65 °C. On the other hand, almost 29 % (XynWu-1) of the maximum activity was found in existence at 1h incubation at 60 °C. For Xyn628, the stability data strongly satisfied that it is a thermostable compared to other xylanase producing *Streptomyces* presented in Table 2.3.

Although, being partially stable at 65 °C, occurrence of greater than 80 % activity at that temperature may be because of high protective effect of the substrate to Xyn628 for optimum time. On the other hand, XynWu-1 stability data seems that it is a thermolabile compared to other xylanase producing *Streptomyces* presented in Table 2.3. Although, being stable at 60 °C, occurrence of the maximum activity at that temperature may be because of protective effect of the substrate to XynWu-1 for optimum time. Many industrial processes are operated at extremes of pH (either acidic and/or alikaline) and at elevated temperatures thus the enzyme must suit the process requirements and must be capable of withstanding such harsh conditions for prolonged periods or at least during the process time which we can find in Xyn628 and XynWu-1. Tolerance to broad range of pH values, a high optimum temperature, and its considerable thermostability makes Xyn628 and XynWu-1 for applicable in various facets of biotechnology such as in biobleaching, xylan hydrolysis, bioethanol production, etc.

3.6 Effect of detergents

3.6.1 Effect of detergents on Xyn628 activity

We have investigated the effect of detergents on the activities of the xylanase and dual nature has been observed. Among all of them, Tween-20 (130 %) enhanced the activity followed by Tween-80 (118 %), Triton XX-100 (109 %), CHAPS (107 %), respectively and polyoxyethylene-4-laurylether (97 %) remained almost unaffected (Table 2.4).

Table 2.4: Effect of detergents on the activity of Xyn628

Detergents	Concentration	Types of ion	Relative Activity [a](%)
None			100±3
Triton X-100	0.25%	Non ionic	77±4
Tween-20	0.25%	Non ionic	92±8
Tween-80	0.25%	Non ionic	90±3
Polyoxylethylene-4 laurylether	0.25%	Non ionic	69±4
Deoxycholic acid	0.25%	Anionic	33±4
Sodium dodecyl sulphate	0.25%	Anionic	7±6
CHAPS	0.25%	Zwitter ionic	85±4

[a] Results are presented as means ± standard deviation (n=3)

Increased in Xyn628 activity by detergents (Triton X-100 and Tween-80) was also reported from *Streptomyces thermocarboxydus* HY-15 [56]. On the other hand, the enzyme activity was highly suppressed by SDS (19 %). In contrast, SDS [57-58] did not influence the xylanases from other origins, such as *Streptomyces* sp. B-12-2, and *Bacillus* sp. 41M-1. Total inactivation due to SDS has already been reported for xylanases of different origins [59]; the inactivation was attributed to the unfolding of enzymes under such condition. However, Xyn628 showed the excellent activity in presence of all types of detergents, except SDS, which makes Xyn628 unique and more applicable in bioindustries.

3.6.2 Effect of detergents on XynWu-1 activity

We have investigated the effect of detergents on the activities of the xylanase and most of the detergents significantly increased its activity.

Among them, Tween-80 (161 %) enhanced the activity followed by CHAPS (157 %), Tween-20 (155 %), Triton X-100 (153 %) and polyoxyethylene-4-laurylether (147 %), respectively (Table 2.5).

Table 2.5: Effect of detergents on the activity of XynWu-1

Detergents (0.25%)	Type of ion	Relative Activity[a] (%)
Triton X - 100	Non - Ionic	153 ± 4.9
Tween - 20	Non - Ionic	155 ± 0.8
Tween - 80	Non - Ionic	161 ± 2.9
Polyoxylethylene-4-laurylether	Non - Ionic	147 + 5.1
Deoxycholic Acid	Anionic	86 ± 4.1
Sodiul Dodecyl Sulphate	Anionic	26 ± 5.9
CHAPS	Zwitter ionic	157 ± 7.5
None	-	100 ± 3.9

[a] Results are presented as means ± standard deviation (n=3)

Increased in XynWu-1 activity by detergents (Triton X-100 and Tween-80) was also reported from *Streptomyces thermocarboxydus* HY-15 [56]. On the other hand the enzyme activity was decreased by DCA (86 %) while the activity was highly suppressed by SDS (26 %). In contrast, SDS [57-58] did not influence the xylanases from other origins, such as *Streptomyces* sp. B-12-2, and *Bacillus* sp. 41M-1. Total inactivation due to SDS has already been reported for xylanases of different origins [59]; the inactivation was attributed to the unfolding of enzymes under such condition. Because of very high influencing effect of detergents on XynWu-1 activity, it can be expected that this enzyme will be a good candidate especially for detergent industry as well as in the bio-industries.

3.7 Effect of metal ions, and other additives

3.7.1 Effect of metal ions, and other additives on Xyn628 activity

Effects of various metal ions, chelating, oxidizing and reducing agents on Xyn628 activity are presented in Table 2.6.

Table 2.6: Effect of metal ions and other additives on the activity of Xyn628

Metal Ion	**Relative Activity[d] (%)**
	1mM
Ca++	105±8
Mg++	37±1
Co++	81±9
Cu++	0±0
Zn++	33±3
K++	91±0
Na++	97±2
Mn++	15±5
Fe++	51±8
Hydrogen peroxide[a]	18±2
Sodium perborate[a]	43±3
β-mercaptoethanol[b]	97±4
1,4-dithiothreitol[b]	128±5
EDTA[c]	18±1
EGTA[c]	26±2
None	100±3

[a]oxidizing agents; [b] reducing agents; [c] chelating agents,

[d] Results are presented as means ± standard deviation (n=3)

The effect of various metal ions was performed at a concentration of 1mM. Xyn628 activity was highly affected by various metal ions, metal chelators (EDTA and EGTA), suggesting its metallo type, similar to our previous report [25]. Xyn628 activity was marginally enhanced by Ca^{2+} (111 %), K^{+} (102 %), and remained almost unaffected by Na^{+} (97 %), Co^{2+} (95 %), respectively. Induced xylanase activity by metal ions suggests that a few metal ions especially Ca^{2+} is required for maintaining the structure stability. However, Cu^{2+} ions are known to catalyze auto-oxidation of cysteines, which leads to the formation of intra molecular and inter molecular disulfide bridges or to the formation of sulphenic acid [60]. Moreover, deactivation of the Xyn628 activity by metal ions was also noticed. Further, the Xyn628 activity was remarkably inhibited by oxidizing agents H_2O_2 and Na-perborate demonstrate that it is oxidant sensitive. On the other side, the effect of β-mercaptoethanol followed by DTT was found unaffected and marginal activation suggesting that cystein residue are not involved in the enzyme activity.

3.7.2 Effect of metal ions, and other additives on XynWu-1 activity

Effects of various metal ions, chelating, oxidizing and reducing agents on XynWu-1 activity are presented in Table 2.7.

Table 2.7: Effect of metal ions and other additives on the activity of XynWu-1

Metal ions	Relative Activity[d]	
	1 mM	5 mM
Ca^{2+}	237 ± 4.33	19.51 ± 2.12
Mg^{2+}	166 ± 6	28.99 ± 4.73
Cu^{2+}	3.11 ± 1.38	0
Co^{2+}	118 ± 1.67	38 ± 2.0
Zn^{2+}	143 ± 3.0	56 ± 7.88
K^{+}	233 ± 6.71	97 ± 9.88
Na^{+}	252 ± 7.21	97 ± 2.13
Mn^{2+}	0	137 ± 6.36
Fe^{2+}	112 ± 3.35	0
Hydrogen peroxide[a]		3 ± 6.1
Sodium perborate[a]		0
β-mercaptoethanol[b]		75 ± 2.6
1,4-dithiothreitol[b]		
EDTA[c]	0	
EGTA[c]	2 ± 3.6	
None	100 ± 3.9	

[a] oxidizing agents; [b] reducing agents; [c] chelating agents,

[d] Results are presented as means ± standard deviation (n=3)

The effect of various metal ions was performed at a concentration of 1mM and 5 mM. The XynWu-1 activity was significantly enhanced by most of the metal ions; Ca^{2+} (237 %), Mg^{2+} (166 %), Co^{2+} (118 %), Zn^{2+} (143 %), K^{+} (233 %), Na^{+} (252 %), and Fe^{2+} (112 %) except Cu^{2+} and Mn^{2+} at a concentration of 1 mM. Induced xylanase activity by metal ions implied that a few metal ions especially Ca^{2+} is required for maintaining the structure stability. However, Cu^{2+} ions are known to catalyse auto-oxidation of cysteines, which leads to the formation of intra molecular and inter molecular disulfide bridges or to the formation of sulphenic acid [60].On the other hand, the effect of metal ions on XynWu-1 was suppressed by most of the metals except Mn^{2+} (137 %) at a concentration of 5 mM. More specifically and interestingly, XynWu-1 activity was enhanced at the concentration of 6 mM (Fig. 2.13) which is unique among xylanases.

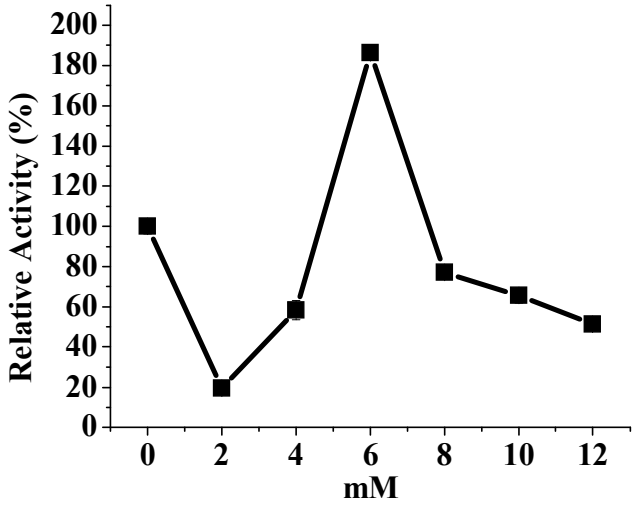

Fig. 2.13: Effect of manganese sulfate concentration on the purified XynWu-1 activity.

Induced XynWu-1 activity by Ca^{2+}, K^{+}, Na^{+}, Co^{2+} and Fe^{2+} were also reported in [21, 61]. Most of the xylanases were found to be inhibited by Mn^{2+} ions, especially from *Streptomyces* [21, 23-24, 26]. However, there are still some exceptions like *Bacillus* sp. strain SPS-0 [62] and *Bacillus amyloliquefaciens* [63], the stimulation of the xylanase activity by Mn^{2+} ion was also found in this study. It may be supposed that the Mn^{2+} ions exert their effect by interacting with some amino acid residue involved in the active site, which as a result may undergo some change in confirmation leading to higher activity, but also higher susceptibility to denaturation at higher temperatures [63]. However, XynWu-1 might be a metallo-protein and metal ions that positively stimulated its activity may be employed as catalysts in trace amounts during application. Metal ions can be involved in enzyme catalysis in a variety of ways: they may accept or donate electrons; they may themselves act as electrophiles; they may mask nucleophiles to prevent unwanted side reactions; they may bring together enzyme and substrate by coordinate bonds; they may hold the reacting groups in the required 3D orientation and they may simply stabilize a catalytically active conformation of the enzyme [35]. Moreover, the present data can be used to determine the amount of enzyme required for different pulp streams with varying metal levels due to the different quality of water used and available. Further, the XynWu-1 activity was completely inhibited by oxidizing agents H_2O_2 and Na-perborate suggests that it is oxidant sensitive, whereas the effect of β-mercaptoethanol followed by DTT was found unaffected suggesting that cystein residue are not involved in the enzyme activity. In case of chelating agents, XynWu-1 activity was completely suppressed by both EDTA and EGTA (Table 2.7). Inhibition of xylanase activity by metal chelators was also reported from *Streptomyces* [26, 39, 56].

3.8. N- terminal amino acid sequences

The first twelve amino acid sequences of the N terminus of Xyn628 were AYIKEVVSRAYM. On the other hand, the first ten amino acid sequences of the N terminus of XynWu-1 were AINVLVAAL. These sequences did not show significant homology with the reported enzymes of similar type. This uniqueness also suggests the novelty of the enzymes.

3.9 Substrate specificity of xylanases

The purified Xyn628 and XynWu-1 enzyme were assayed with varieties of substrates and the highest xylanases activity observed with commercially available beechwood xylan compared with birchwood xylan (Table 2.8).

Table 2.8: Evaluation of substrate specificity and cellulase activity on Xylanases

Substrate (0.5%)	Relative Activity (%)	
	Xyn628	**XynWu-1**
Beechwood xylan	100±2	100±1
Birchwood xylan	53.09±1	59±2
Na-CMC	0.66±4	1.00±5
Avicel 101	0.57±2	0.50±1
pNPC	0.47±5	0±2
pNPG	0.57±1	0±1

The enzymes didn't showed any cellulase activity (Table 2.8) with Avicel101, Sodium Carboxymethyl cellulose (Na-CMC), p-nitrophenyl glucopyranoside (pNPG) and p-nitrophenyl -β-D-celobioside (pNPC). These results demonstrate that Xyn628 and XynWu-1 are cellulase free

xylanase, similar to xylanases reported in *Streptomyces*, [21, 64] which is one of the major properties as a biobleaching agent for paper and pulp industries.

3.10 Kinetic parameter of xylanases

The kinetic constants (K_m) and (V_{max}) of Xyn628 were determined by Eadie-Hofstee Plot using beech wood xylan were 3.1 ± 0.5 mg/mL and 313 ± 13 mmoL/min mg, respectively. K_m value of Xyn628 is slightly lower than *S. rameus* L2001 [23] but its higher V_{max} value suggests that it is more efficient than *S.* sp. CS802 [25]. On the other hand, the kinetic constants (K_m) and (V_{max}) of XynWu-1 were determined by Eadie-Hofstee Plot using Beech wood xylan were 1.7 ± 0.04 mg/mL and 742 ± 2.5 mmoL/min mg, respectively. Lower K_m and higher V_{max} value of XynWu-1 are comparable with other xylanase from *S. rameus* L2001 (5.3 ± 0.04 mg/mL) [21] and *Streptomyces* sp. CS802 (338 ± 66 mmoL/min mg) [25]. Thus, suggests that XynWu-1 has high affinity for the substrate and more efficient at decomposing beechwood xylan.

3.11 Mode of hydrolysis and potential application of xylanases in xylooligosaccharides production

3.11.1 Mode of hydrolysis and potential application of Xyn628 in xylooligosaccharides production

As depicted in fig.2.14, Xyn628 produced mainly xylobiose (X2) and xylotriose (X3) followed by series of oligosaccharides indicating it as endoxylanase in nature. Generally, in the case of exo-acting enzymes, only monomer or dimer accumulates throughout the enzyme reaction. In this study, xylobiose, and several xylooligosaccharides were produced by the action of enzyme,

demonstrating that Xyn628 is an endo-type xylanase and similar type of results also reported in xylanases from *Streptomyces* [25-26].

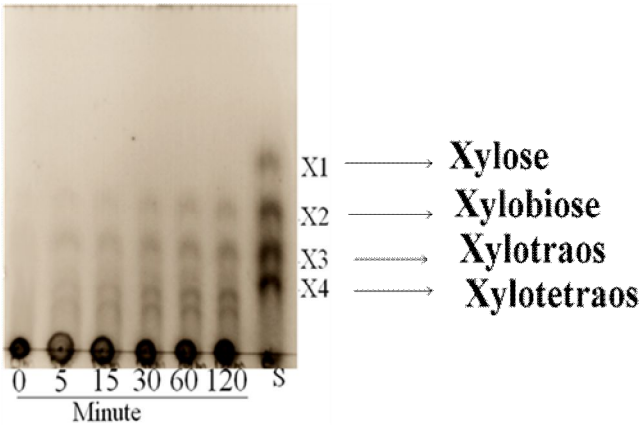

Fig. 2.14: Time-course of hydrolysis of xylan by the Xyn628.

In recent trends, xylooligosaccharides are of great interest in the food industry as well as in modern medicinal applications due to their prebiotic potential. Specially, xylobiose and xylotriose can be directly used in food ingredients because these are known to positively modulate the intestinal microflora and suppress the growth of pathogenic microbiota [65]. However, during the process of xylooligosaccharide production, high xylobiose and low xylose is desirable because the hydrolysate containing more than 12 % of xylose is of low efficacy for the industrial purpose [66]. Furthermore, Xyn628 more intensely degraded wheat bran pulp and corncob powder even at very low dose, 20 U/g, as analyzed by scanning electron micrographs (Fig. 2.15). The degradation was dose dependent, and clear difference can be noticed among buffer-treated sample and 20 or 2000 U/g enzyme-treated sample. The enzyme was very active even at low dose (20 U/g), which is very encouraging.

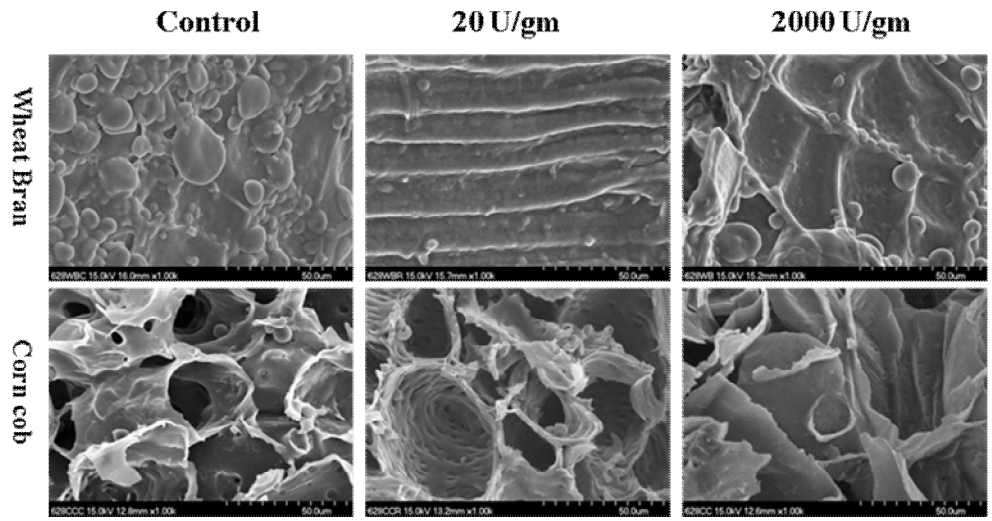

Fig. 2.15: Scanning electron micrographs (SEM) of wheat bran pulp and corncob powder degradation by Xyn628 (magnification 1,000)

The result demonstrates that Xyn628 is suitable for the degradation of agro-residues such as wheat bran, corncob and many other xylan rich agro-residues, and thus can be utilized for the production of value-added products such as xylooligosaccharides, bioethanol, etc. Basically, successful degradation of agro-wastes by Xyn628 suggests that the production cost of xylooligosaccharides can be hopefully reduced significantly by utilizing cheap, renewable, readily available, and unnecessary occupying xylan rich agro-residues; resulting keeps our environment more clean and pollution free. More importantly, low molecular weight xylanases as bleaching agents in pulp and paper industry are desirable, since they can easily penetrate into the re-precipitated xylan on the surface of pulps. This alleviates the problem of xylan barrier on the surface of lignin containing pulp during subsequent chemical bleaching steps [17]. However, lack of cellulase activity is also important for the production of xylooligosaccharides [26].

Furthermore, several reports already published for an ideal xylanase to be useful for biobleaching, it must be active and stable at high temperatures and under alkaline conditions, and must also have free from cellulase activity [20, 26]. Therefore, Xyn628 possesses these features and, thus, should be of interest of various potential bioindustrial applications.

3.11.2 Mode of hydrolysis and potential application of XynWu-1 in xylooligosaccharides production

Fig. 2.16 showed the time-course hydrolysis of beechwood xylan by XynWu-1. Hydrolysis of beechwood xylan was found to yield xylose, xylobiose and xylotetraose as the major products followed by plausible production of xylopentose. Its mechanism of action on xylan indicates that it is an endo-xylanase and almost similar type of pattern also reported in [24], [39].

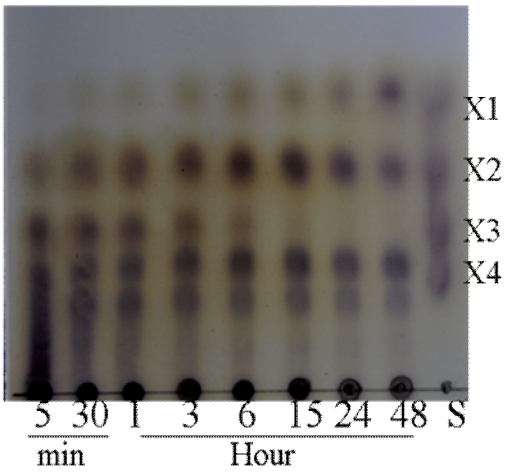

Fig. 2.16: Time-course of hydrolysis of xylan by XynWu-1. S, mixture of Xylose (X1), Xylobiose (X2), Xylotriose (X3) and Xylotetraose (X4).

Hydrolysis by xylanase is the major process for the production of xylooligosaccharides. Presently, xylooligosaccharides are of great interest in the food industry as well as in modern medicinal applications due to their prebiotic potential. The prebiotic effect of xylooligosaccharides modulates the colonic microbial flora, likely stimulating the growth of beneficial bacteria and suppresses the growth of pathogenic microbiota [67]. In addition, xylooligosaccharides can be used as a source of xylose for the production of xylitol or for the preparation of ethers and esters which can be used as thermoplastic compounds for water-soluble films, coatings, and capsules which has wide applications in the pharmaceutical industry as excipients [68]. Furthermore, as depicted in fig 2.17, XynWu-1 more intensely degraded corncob and wheat bran pulp even at very low dose, 20 U/g, as analyzed by scanning electron micrographs.

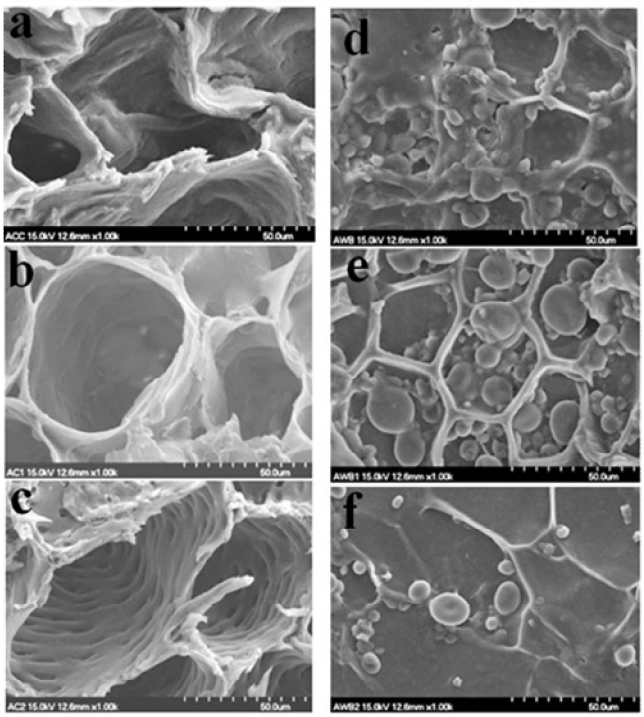

Fig. 2.17: Scanning electron micrographs (SEM) of wheat bran and corncob powder degradation by XynWu-1 (magnification 1,000X). 1, Corncob; 2, Wheat bran; a & d, control; b & e, 20 U/g; c & f, 2000 U/g.

The result suggests that XynWu-1 is suitable for the degradation of agro-residues such as corncob, wheat bran and many other xylan rich agro-residues and thus can be utilized for the production of value-added products such as xylooligosaccharides. Actually, successful degradation of agro-wastes by XynWu-1 suggests that the production cost of xylooligosaccharides can be hopefully reduced significantly by utilizing cheap, renewable, readily available, and unnecessary occupying xylan rich agro-residues. However, lack of cellulase activity is also important for the production of xylooligosaccharides [26] and XynWu-1 was also assessed to evaluate any cellulase activity was present using various substrates and found that cellulase activity was completely absent. Besides, several reports already published for an ideal xylanase to be useful for biobleaching, it must be active and stable at high temperatures and under alkaline conditions, and it must also have lack of cellulase activity [20, 26]. Therefore, XynWu-1 possesses these features and, thus, should be of interest of various bioindustrial applications.

4. CONCLUSIONS

In this study, potent xylan degrading enzymes were isolated and identified as *Streptomyces* sp. CS 628 and CSWu-1. Both extracellular xylanases, Xyn628 and XynWu-1, were purified and biochemically characterized. Xyn628 showed lowest molecular weight among the so far reported *Streptomyces* xylanase. This feature, incorporating with unique N-terminal sequences, it might be a novel in its kind. The xylanases were optimally active at pH 11.0 and stable in a wide range of pH 5 to 13, suggesting that these are alkaline-tolerant xylanase. Optimum temperature of Xyn628 and XynWu-1 were 60 °C and stable up to 65 °C and 55 °C, respectively. Xyn628 and XynWu-1 activity were enhanced by most of the detergents. Especially, XynWu-1 activity was enhanced by most of the metal ions. Even though, XynWu-1 activity at higher molar concentration by Mn^{2+} ion suggested unique among *Streptomyces* which might be a metalloprotein type xylanase. Xyn628 and XynWu-1 produced series of xylooligosaccharides as the major products from the xylan, suggesting both are endoxylanase in nature. Furthermore, Xyn628 and Xynwu-1 lacks cellulase activity and efficiently degrades corncobs and wheat bran. Successful degradation of agro-waste corncob and wheat bran by Xyn628 and XynWu-1, which might useful in the production of value-aded xylooligosaccharides by considering low economic cost and promise to keeps our environment more clean and pollution free. Based on the above mentioned characteristics, Xyn628 and Xynwu-1 is highly likely to be useful for various biotechnological applications, such as biobleaching in paper and pulp industries, production of xylooligosaccharides for prebiotic in food and pharmaceutical industry, potential application in biofuel and textile industries as well as waste treatment with appropriate utilization of agro-waste products.

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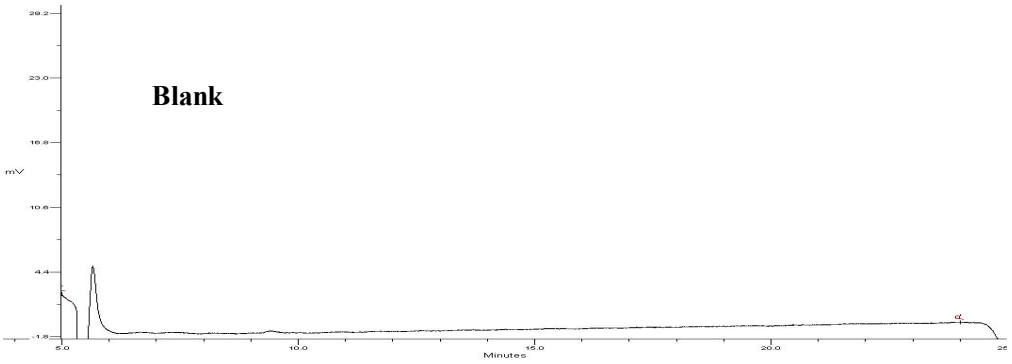
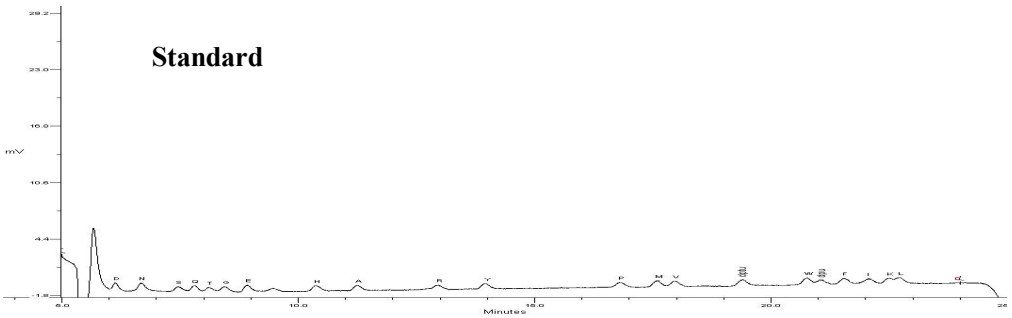
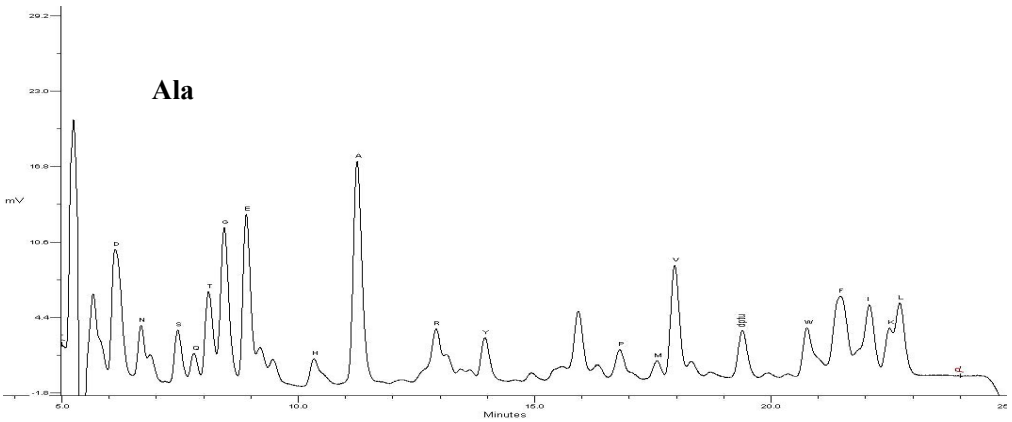
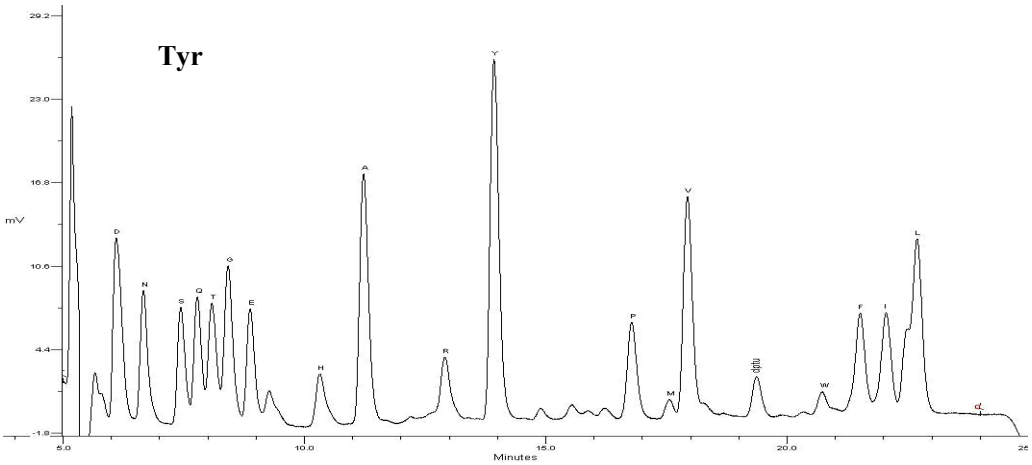
Appendix 1: N-terminal sequences of Xyn628





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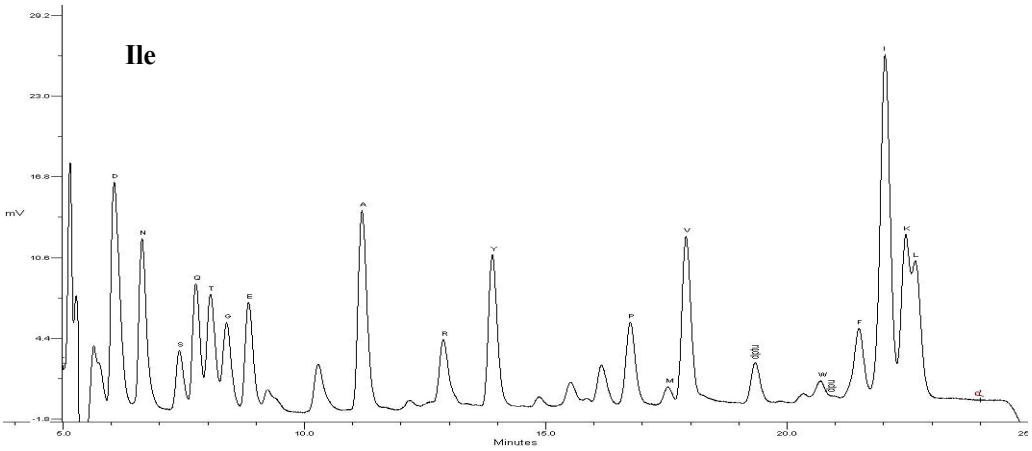
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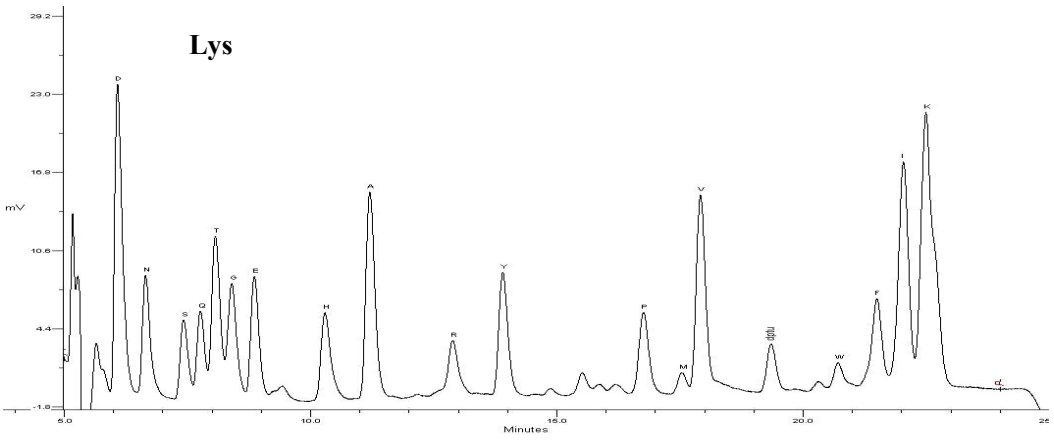
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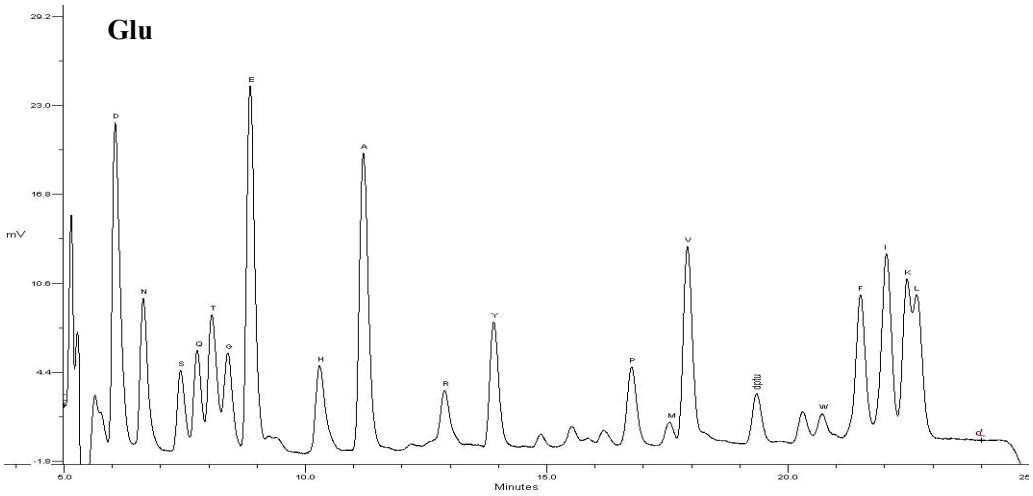
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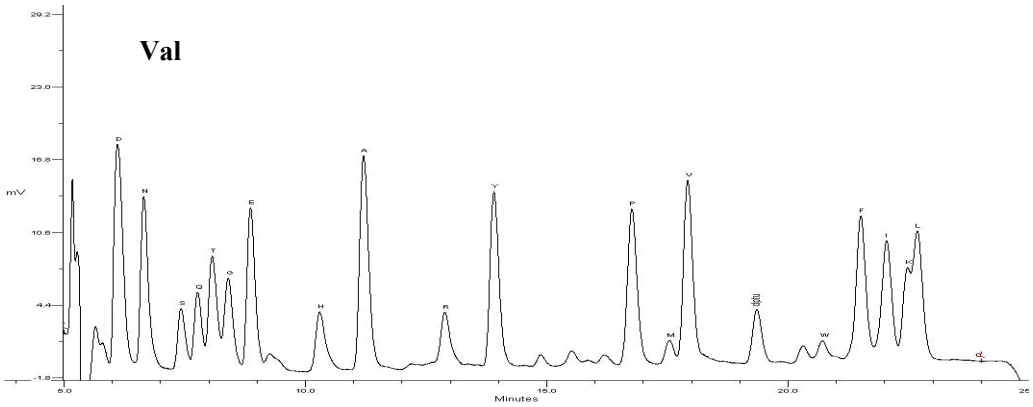
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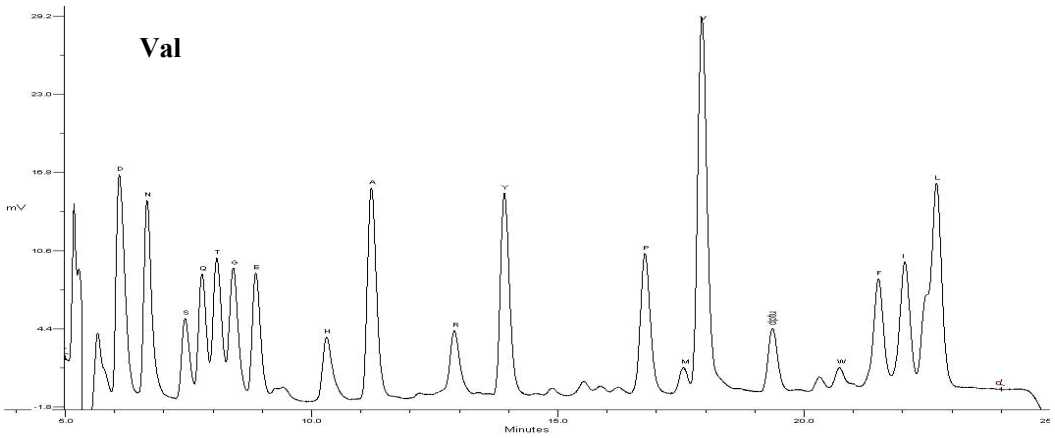
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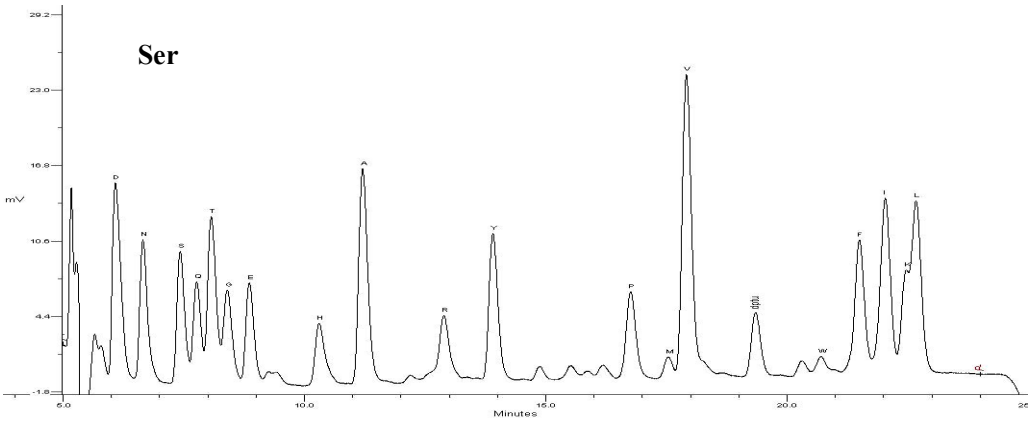
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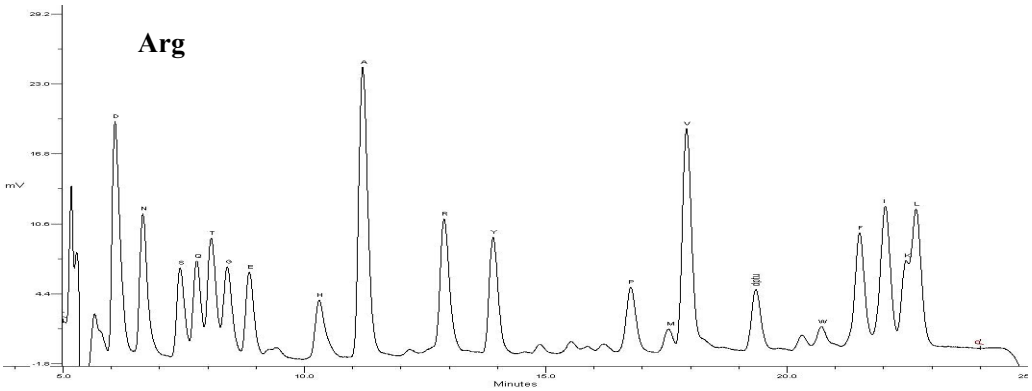
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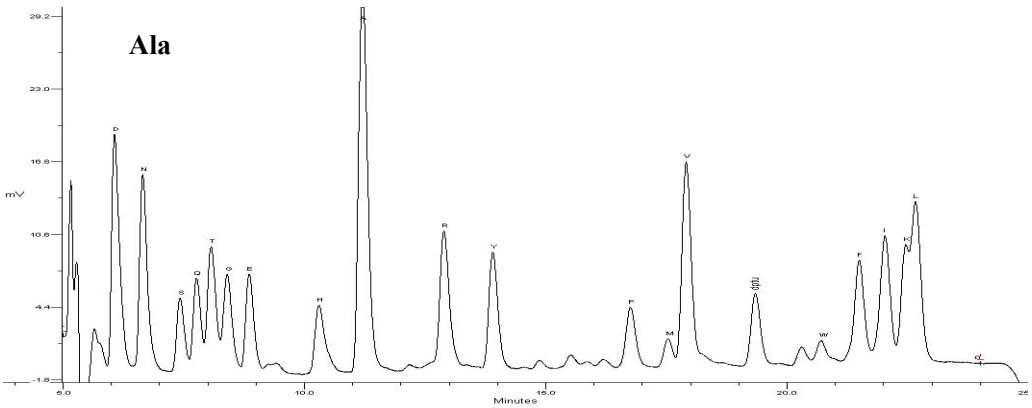
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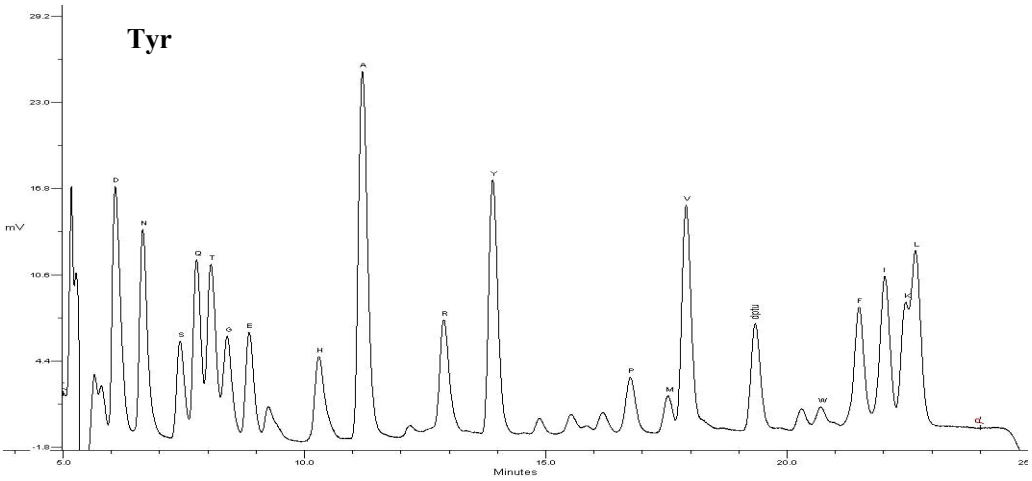
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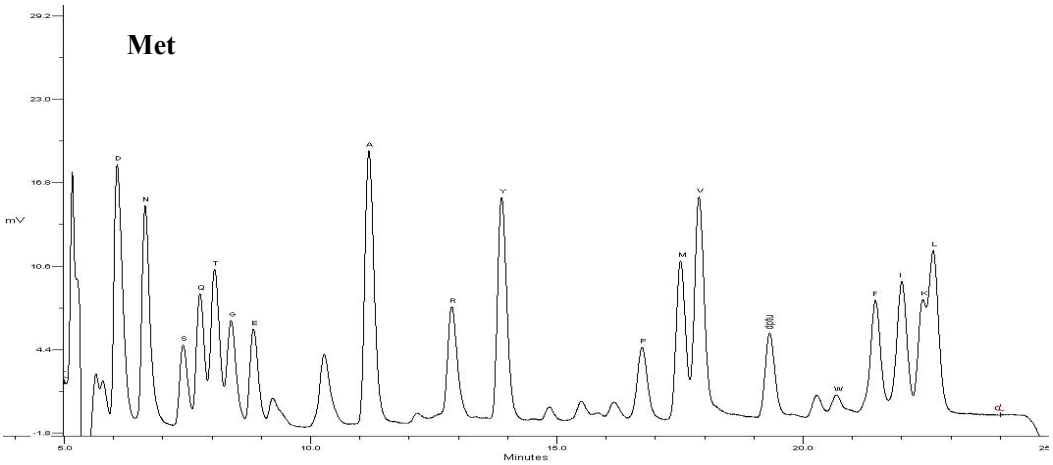
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Minutes
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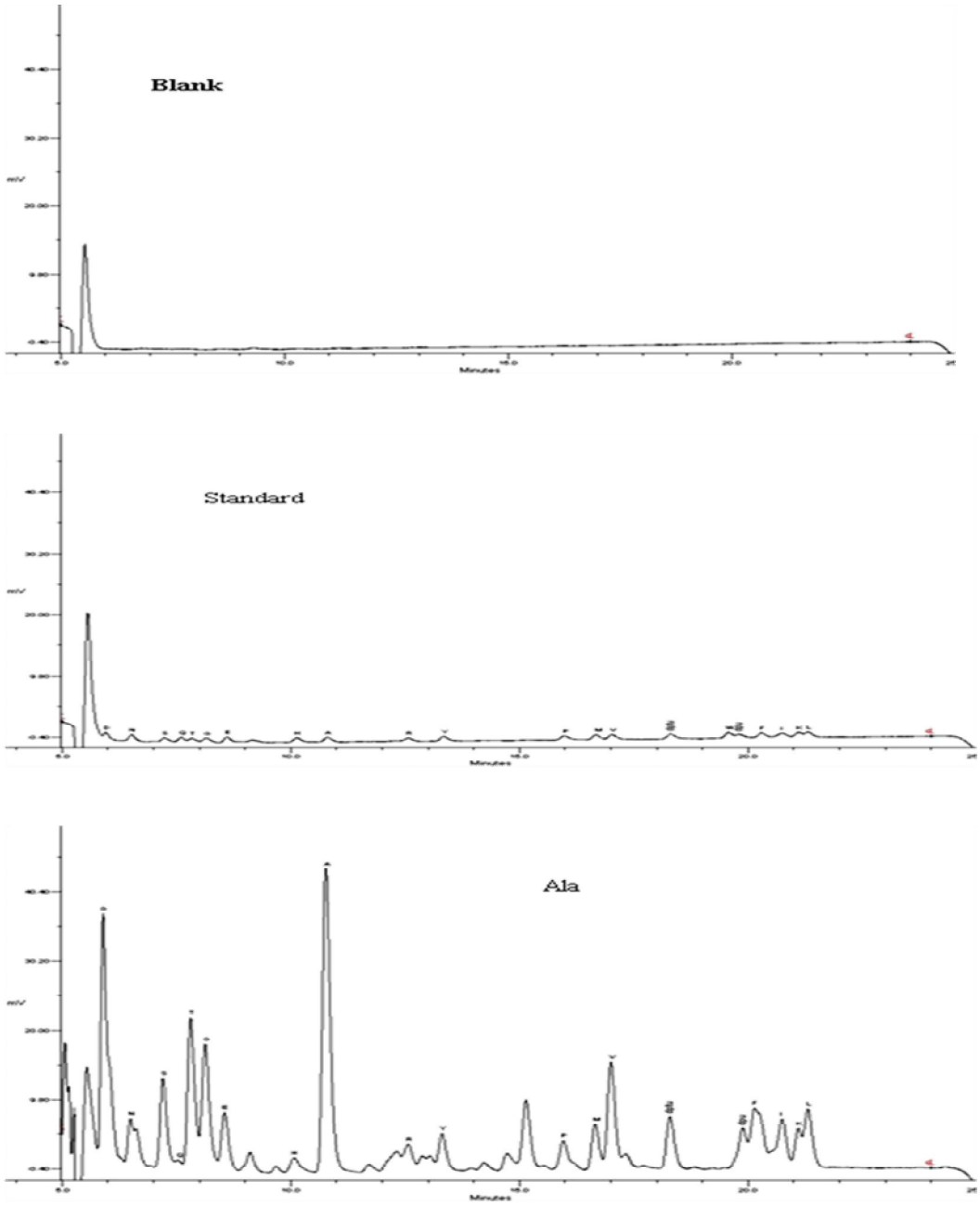
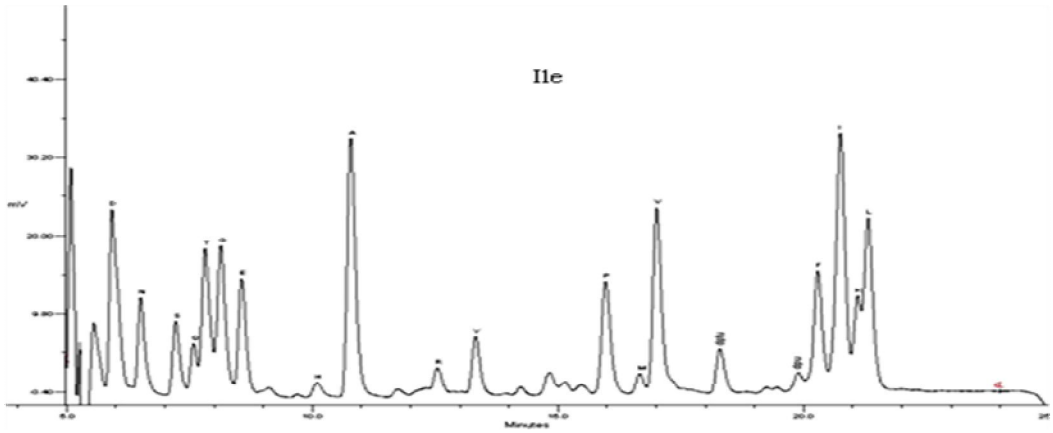
Appendix 2: N-terminal sequences of XynWu-1

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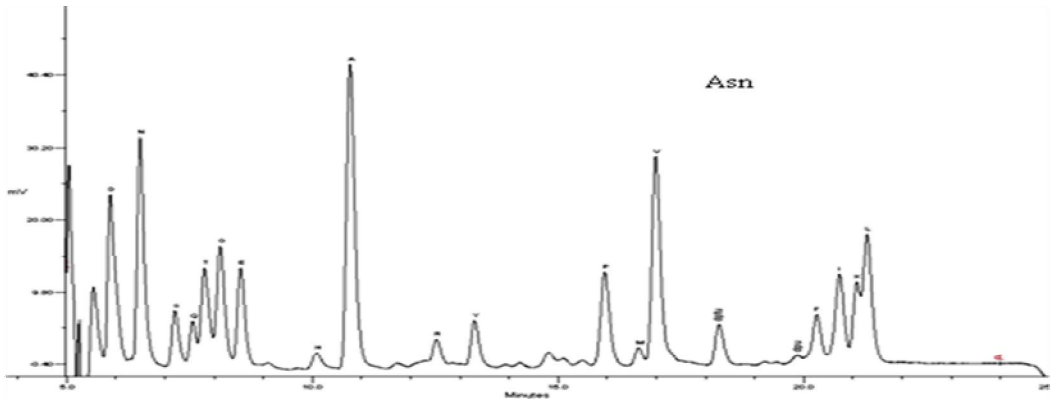
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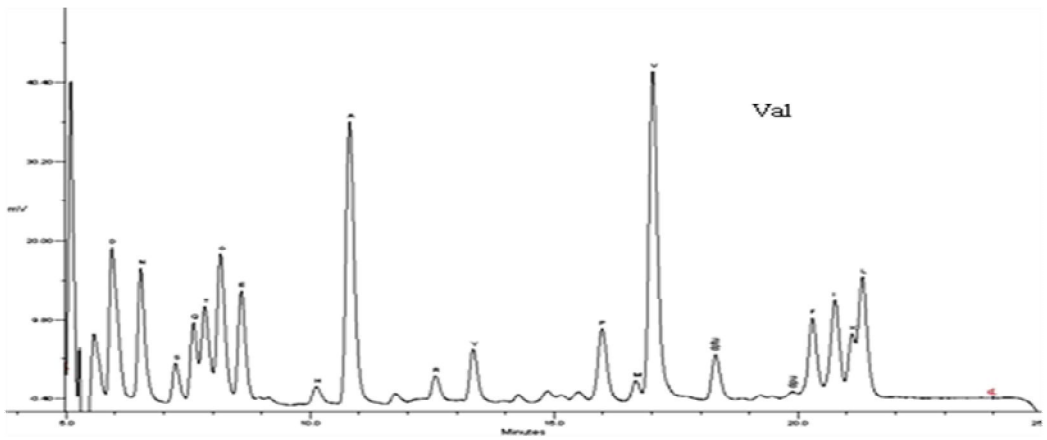
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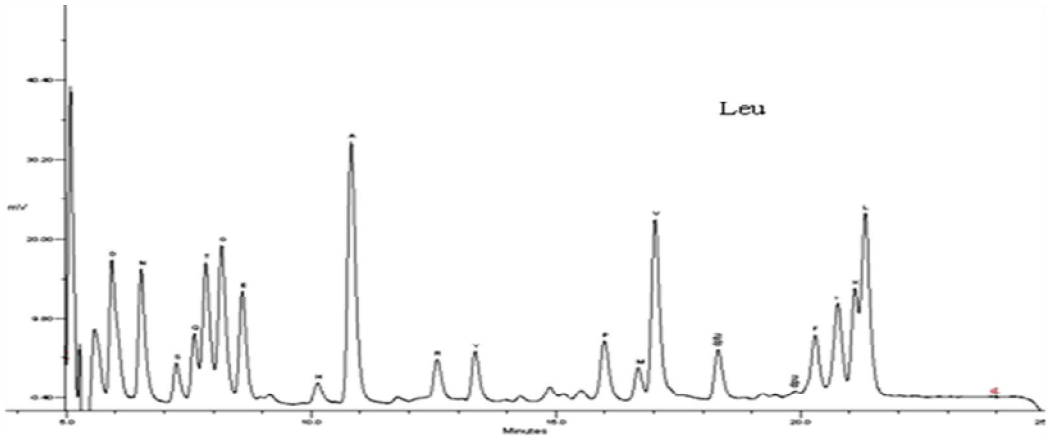
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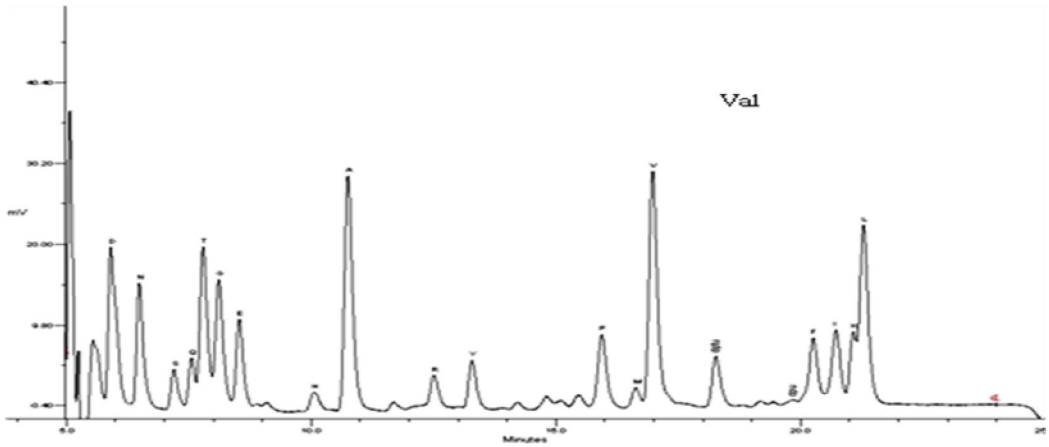
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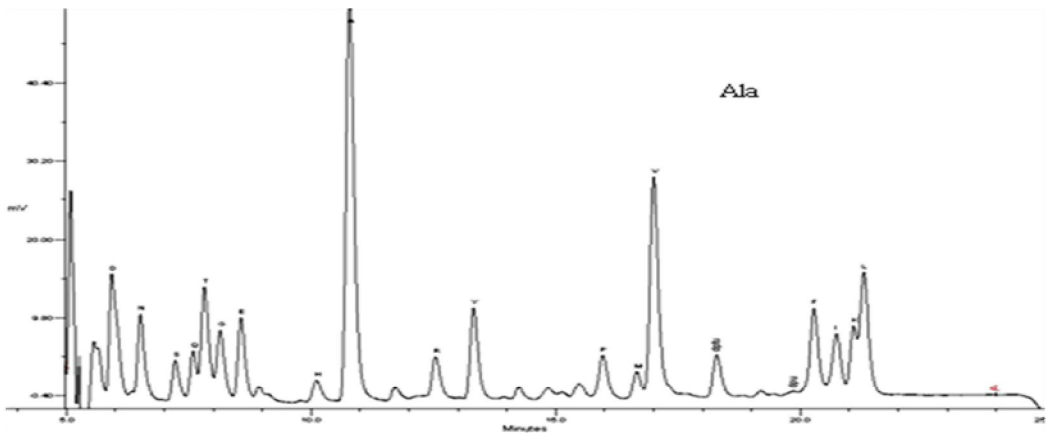
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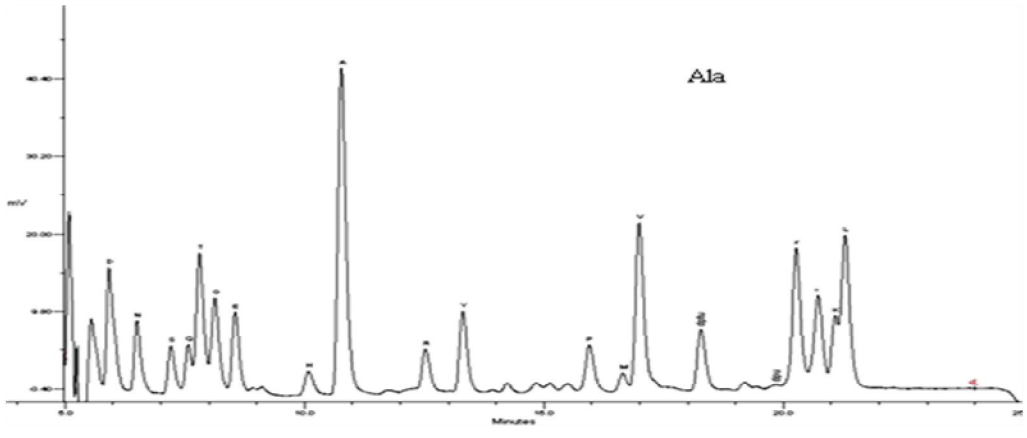
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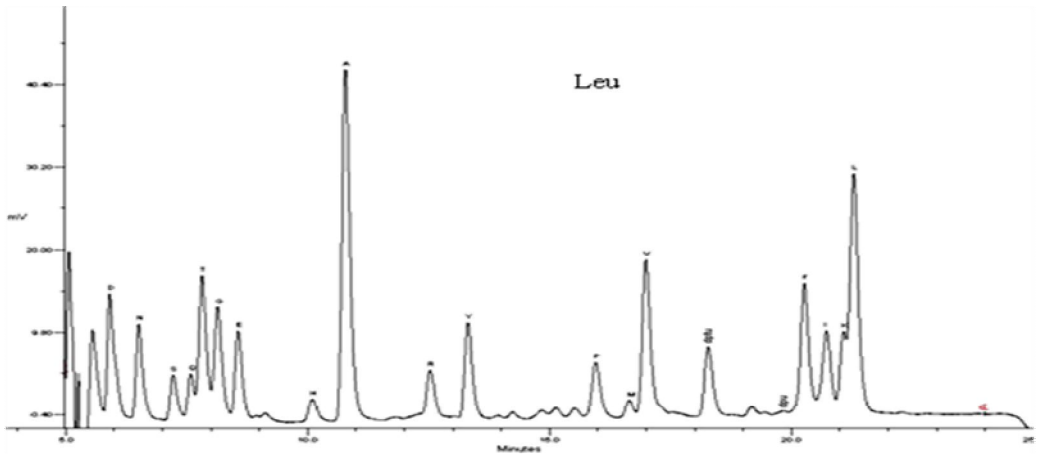
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Minutes

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