PRODUCTION AND CHARACTERIZATION OF XYLANASES AND THEIR APPLICATIONS IN BLEACHING

Ph.D. THESIS

by

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ROORKEE – 247 667 (INDIA)
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A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY
in
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ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled **"Production and Characterization of Xylanases and their Applications in Bleaching"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Paper Technology of the Indian Institute of Technology Roorkee, is an authentic record of my own work carried out at Department of Paper Technology during a period from July, 2009 to September, 2015 under the supervision of Dr. Dharm Dutt, Professor, Department of Paper technology, Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(Archana Gautam)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Date: May , 2016 **(Dharm Dutt)**
Supervisor

The Ph.D. Viva-Voce Examination of **Mrs. Archana Gautam**, Research Scholar, has been held on…….

Signature of Supervisor **Signature of External Examiner**

Chairman, SRC/CRC **Head of the Department/Centre**

ABSTRACT

Annual production of paper, paperboard and newsprint in India is 10.11 million tonnes while consumption of paper, paperboard and newsprint in the country is 11.15 million tonnes/ annum till 2012. Indian pulp and paper industry will require 22.0 million tonnes of paper and paperboard till 2025 with an average growth rate of 7.8% per annum. In, India the major raw materials for paper, paperboard and newsprint production were forest-based (31%), agricultural residues (22%) and secondary fibres (47%) in year 2011. There is an acute shortage of forest-based raw materials globally. Natural forests, planted timberlands and agro-residues are the main sources of lignocellulosic raw materials for paper production. Due to increased demand of pulp products, there will be more harvesting pressure on these resources. To mitigate the gap between demand and supply, various fast-growing and high yielding annual and perennial, non-conventional and cellulosic raw materials have been identified and assessed for their suitability for pulp production.

The process of papermaking from virgin fibre requires pulping and bleaching chemicals for delignification of raw material. Due to growing environmental concerns and legislative pressures the pulp and paper industry is forced to modify its current pulping, bleaching and effluent treatment technologies. Today, the pulp and paper industry is adopting the eco-friendly technologies to reduce the pollution and to meet the challenges of globalization. It is evident that about 20 to 25% degradation of carbohydrates occurs during pulping and chlorine-based bleaching technologies exerted an impact to our eco-system. Therefore, efforts should be made to develop such pulping technologies which mitigate the carbohydrate degradation during pulping. The selection of molecular chlorine free bleaching sequences, substitution of molecular chlorine with less hazardous bleaching chemicals or mitigation of total chlorine demand during bleaching by extended delignification should be the aim of future studies. With these goals, ethanol-soda pulping process for *E. binata* was developed to minimize carbohydrate degradation during pulping and compared with other conventional pulping methods suitable for non-woods or grasses. Similarly, all the three major bleaching processes like conventional, ECF and TCF bleaching sequences were studied and effect of enzymes on pulp yield, optical properties, mechanical strength properties and effluent characteristics were studied.

Enzymes are the biocatalysts which can offer potential advantages to paper industry like in biopulping, biobleaching, biorefining, biodeinking, and effluent treatment. Enzymes can serve as a promising biotechnological tool being the best alternative to polluting chemical technologies. Xylanases are gaining popularity as the catabolic agent for delignification in the bleaching process.

This work depicted the production of xylanase by under solid-state fermentation (SSF) conditions using various agro-waste materials as the carbon sources. Morphological and proximate chemical studies of *Eulaliopsis binata* were carried out to evaluate its papermaking potential. Pulping conditions were optimized to obtain maximum pulp yield with better optical and physical strength properties. The enzyme produced in crude form was used for prebleaching of ethanol-soda *E. binata* pulp.

In this work, out of 20 isolates obtained after screening for xylanase activity, two isolates namely ARC-11 and ARC-12 were selected for further studies. Xylanase producing fungi were identified as *Schizophyllum commune* ARC-11 and *Aspergillus flavus* ARC-12 isolated from wood and soil samples, respectively. The efforts were made to find out the optimum fermentation conditions for enhanced production of xylanase. Recent studies are focused on SSF with special reference to enzyme production using different agro-industrial wastes. Eleven agro-residues were tested as the carbon sources for xylanase production for both the fungal strains. In case of *Schizophyllum commune* ARC-11, the best carbon source for enzyme production was rice straw (4288.36 IU/gds) while pearl millet stover (1345.44 IU/gds) for *Aspergillus flavus* ARC-12. Depending on the fungal growth, incubation time range tested for *Schizophyllum commune* ARC-11 was 1-12 days and 12-96 hours for *Aspergillus flavus* ARC-12. Xylanase production was found to be the maximum (5199.02 IU/gds) after 8^{th} day of incubation for *Schizophyllum commune* ARC-11 and 2^{nd} day of incubation for *Aspergillus flavus* ARC-12 (1424.69 IU/gds). To optimize the incubation temperature, both the fungal strains were tested under different temperature ranging from 26-46°C keeping at an interval of 4°C. An incubation temperature of 30°C resulted maximum xylanase production for *Schizophyllum commune* ARC-11 (5358.93 IU/gds) and *Aspergillus flavus* ARC-12 (1431.19 IU/gds). An Initial pH of 7.0 was found to be the optimum for *Schizophyllum commune* ARC-11 (6340.71 IU/gds) and 6.0 for *Aspergillus flavus* ARC-12 (1663.72 IU/gds). Initial moisture

content of 70% was observed to be the optimum for *Schizophyllum commune* ARC-11 (6721.96 IU/gds) and 77.5% for *Aspergillus flavus* ARC-12 (1699.50 IU/gds). The nature and amount of nitrogen sources affects the enzyme production. Ammonium sulphate (0.08% N) for *Schizophyllum commune* ARC-11 and beef extract (1.2%, w/v) for *Aspergillus flavus* ARC-12, gave maximum xylanase production of 8591.38 and 2219.85 IU/gds, respectively. Among various surfactants tested for xylanase production Tween-20 (0.10% w/v) produced maximum xylanase activity, 10196.53 IU/gds for *Schizophyllum commune* ARC-11 and Tween-60 (0.10%) produced maximum xylanase activity (2539.54 IU/gds) for *Aspergillus flavus* ARC-12. Characterization of the xylanase of *Schizophyllum commune* ARC-11 clearly showed the activity in a wide range of pH 4.0-7.0 (optimum 5.0) and temperature range of 35-60°C (optimum at 55 °C). Xylanase from *Aspergillus flavus* ARC-12 was observed to have a broad pH range from 4.0 to 8.0 (optimum pH 6.0) and temperature range from 35-65°C (optimum 50°C). The fraction of 50-70% ammonium sulphate precipitation gave a yield of 41.86% with 2.75 fold purification for xylanase from *Schizophyllum commune* ARC-11. With the same fraction of 50-70% ammonium sulphate precipitation xylanase from *Aspergillus flavus* ARC-12 gave a yield of 45.05% with 2.85 fold purification.

The detailed morphological and anatomical features of *E. binata* were determined. The results of proximate chemical analysis revealed that *E. binata* contain higher holocellulose (73.1%) and α-cellulose contents (46.0%) respectively, which are directly related to good strength and high pulp yield for papermaking. Optimization studies were performed for alkali charge (active alkali as Na_2O), cooking temperature, time and moisture content and its effects on unscreened pulp yield, screened pulp yield, kappa number and rejects were studied. *E. binata* produced a screened pulp yield of 43.58% with kappa number 17.38, and rejects 0.88% at optimum cooking conditions like active alkali charge 12% (as Na_2O)), maximum cooking time 120 min and temperature 130 °C. A comparative study of soda, ethanol-soda and bio-soda pulping was carried out. Addition of ethanol (30%), along with alkali at optimum conditions gave maximum screened pulp yield (47.48%) compared to 42.76 and 43.58% for bio-soda and soda pulping respectively. The kappa number was reduced by 7.19 and 7.24% during ethanol-soda and bio-soda pulping of *E. binata* compared to soda pulping. Mechanical strength properties were determined for all the three types of pulps at a beating level of 35±1 °SR.

Improvement in strength properties was observed in case of ethanol-soda and bio-soda pulping processes compared to soda pulping. The addition of 30% ethanol, improved pulp brightness (6.6%), tensile index (32.18%), burst index (35.40%) and double fold numbers (77.31%) compared to soda pulping. Tear index decreased by 9.95% in ethanol-soda pulping of *E. binata* compared to soda pulping.

The main objective of bleaching is to make all the chemical pulps whiter or brighter without compromising the strength of the pulps. The major source of dioxins in the pulp and paper industry is the bleaching process in which Cl_2 is used as chemical of choice. Lignin removal is more selective in the chlorination and extraction stages than in the pulping process. The crude xylanases produced by *Schizophyllum commune* ARC-11 and *Aspergillus flavus* ARC-12 were successfully used in bio-bleaching of *E. binata* ethanol-soda pulp. A xylanase dose of 10 IU/g (OD pulp basis), reaction time 120 min and consistency 10% were found optimum for pre-bleaching treatment by xylanases from *Schizophyllum commune* ARC-11 and *Aspergillus flavus* ARC-12. Xylanase pretreated pulps showed an improvement in brightness during all the bleaching sequences, compared to untreated pulps. In conventional bleaching, chlorine demand mitigated by 23.50 and 24.50% using xylanases from *Aspergillus flavus* ARC-12 and *Schizophyllum commune* ARC-11 respectively in X_1ECEHH and X_2ECEHH bleaching sequences compared to CEHH. Due to reduction in chemical demand release of AOX in effluents reduced by 21.49 and 28.50% using xylanase from *Aspergillus flavus* ARC-12 and *Schizophyllum commune* ARC-11 respectively compared to control. Brightness and tear index improved in case X_1DEPP and X_2DEPP compared to DEPP at the same chemical dose. Brightness was improved by 2.8 and 1.4% (ISO) during X_1QOPP and X_2QOPP bleaching sequences compared to QOPP in TCF bleaching. Finally we it is concluded that the sequence X_1DEDP was found most effective in bleaching of pulp of *E. binata*.

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(Archana Gautam)

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Abbreviations

°C	Degree Celsius
°SR	Degrees schopper reigler
µm	Micrometer
AOX	Adsorbable organic halogens
BSA	Bovine serum albumin
cm	Centimeter
CMC	Carboxy methyl cellulose
COD	Chemical oxygen demand
Cps	Centipoise
CSF	Canadian standard freeness
ECF	Elemental chlorine free
EDTA	Ethylenediaminetetraacetic acid
g	gram
gds	gram dry substrate
gsm	Gram per meter square
h	hour
ha	Hectare
HexA	Hexeneuronic acid
ISO	International organization of standardization
ITS	Internal transcribed spacer
IU	International units

kg	Kilogram
kJ	Kilojoule
kPa	Kilopascal
l	Litre
LCC	Lignin carbohydrate complex
m	Meter
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimole
MT	Million tonnes
N	Newton
nm	Nanometer
OD	Oven dry
PDA	Potato dextrose agar
PTU	Platinum cobalt unit
s	second
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
SmF	Submerged fermentation
SSF	Solid-state fermentation
t	Tonne

TCF	Total chlorine free
TOCl	Total organic chlorides
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
XRD	X-ray diffraction
TCDD	Tetrachlorodibenzodioxin
TCDF	Tetrachlorodibenzofuran
rpm	Revolution per minute

CHAPTER 1

INTRODUCTION

Paper plays a vital role in the socio-economical development of any country. Increasing paper consumption due to growth of the world's population along with enlarging diversity of paper applications are putting more harvesting pressure on limited natural resources of the planet. World consumption of paper and paperboard in 1963 was 165 million tonnes which went up approximately 253 million tonnes in 1993. Global production of paper and paperboard in 2013 was 397 million tonnes. Figure 1.1 shows, trend in the production of fibre furnishes (paper and paperboard) in last five years. The production of fibre furnish in year 2010 increased up to 397 million tonnes compared to 377 million tonnes in 2009. Over a period of 2010-2013 production of fibre furnish was quite stable at the level about 400 million tonnes [1-3].

Annual production of paper, paperboard and newsprint in India is 10.11 million tonnes while consumption of paper, paperboard and newsprint in the country is 11.15 million tonnes/ annum till 2012. Indian pulp and paper industry will require 22.0 million tonnes of paper and paperboard till 2025 with an average growth rate of 7.8% per annum [4]. In, India the major raw materials for paper, paperboard and newsprint production were forest (31%), agricultural residues (22%) and secondary fibres (47%) in year 2011 (Figure 1.2) [5].

There is an acute shortage of forest-based and conventional raw materials globally. Natural forests, planted timberlands and agro-residues are the main sources of lignocellulosic raw materials for paper production [6, 7]. Due to increased demand of pulp products, there will be more harvesting pressure on these resources. Wood-based segment of the paper industry has considerably shrunk, as in 1970, forest-based segment of Indian paper industry was 84% which had been reduced to 31% in 2011 [5]. 67.8 million hectare of forest cover in India is reported which is 20.6% of the country's surface area and it translates into a per capita forest area of only 0.8 ha/person, one of the lowest in the world [8]. The per capita consumption of paper and paperboard and newsprint in India was 9.3 kg in 2010 as against the world average of 56.7 kg (Figure 1.3). In India the paper consumption is predominantly domestic and the demand is driven by GDP growth [9]. There are 759 pulp and paper mills with have an installed capacity of 12.7 million tonnes and producing 10.11 million tonnes of paper and paperboards which is

2.52% of the total world production (Table 1.1) [9]. Severe exploitation of forests may be detrimental for environment. Moreover, cost of available wood is increasing due to higher demand, higher cost of harvesting and increasing stumpage fee. The question is how to meet the increasing global demand of 2-3% for paper and paperboard [3]. Therefore, inadequate supply of wood fibre due to rapid depletion of forest wealth, forces the dependence of paper industry on other alternatives. Non-woody plants, agricultural residues and waste paper are such alternatives which may meet the demand for pulp and paper industry [10-13].

However, paper making from recovered fibre requires less energy in comparison to virgin fibre [14] and every metric tonne of recycled fibre saves trees, water, electricity and reduce air pollution [15-18]; but, fibre length and strength properties decreases at every time of recycling therefore, recycled fibre can't replace the need of virgin fibre completely. The utilization of recycled fibre for paper, paperboard and newsprint production increases rapidly but recycled fibre has limited recyclability (average 2.4 times worldwide). Therefore, virgin fibre always requires to fulfill the demand of paper, paperboard and newsprint [19, 20].

Due to global shortage of forest based raw materials non-wood fibres have become the one of the potential alternatives in 21st century. Non-wood fibres are the cellulosic plant materials which can be utilized for the production of pulp for paper making. There are several non-wood plants such as wheat straw, rice straw, bagasse, bamboo, kenaf, hemp, jute, sisal, abaca, cotton linter, cotton stalk etc have been used for paper making all over the world [21-26]. Average agricultural residues contain 19–27% hemicellulose, 32–47% cellulose, 10–24% lignin, and 10–24 % ash content [27]. India produces large quantity of agro-residues and annual potential of agro-fibres in India is given in Table 1.2. Agro-residues are also used for animal feed, biomass fuel production, and composting which competes with pulp and paper industry for availability the availability of agro-residues [28]. The need of good quality of fibre compels the paper industry to search other alternate resources of fibre. Many fast growing annual and perennials plants have been identified, cultivated and studied for their suitability for pulp and paper industry [29]. Different grasses such as *Arundo donax* [30], *Ipomea carnea* and *Cannabis sativa* [31], dogs tooth grass (*Chenopodium album*) [29], Lemon grass (*Cymbopogon flexuosus*) and Sofia grass (*Cymbopogon martini*) [32], *Phragmites karka* [33], Switchgrass and Elephant grass [34] have assessed for pulp and paper production.

Kenaf (*Hibiscus cannabinus*) is used as raw material for papermaking in both developing as well as developed countries. Pulp extracted from kenaf has desirable properties for paper making and strength properties of kenaf are comparable to coniferous wood pulps. Kenaf plant gives an excellent average annual yield which is twice compared to fast growing softwoods (Table 1.3). Kenaf can be harvested for several months and pulp yield is also high for this plant [3, 35-37]. Sugarcane bagasse is another suitable raw material for pulp and paper production. Sugarcane bagasse is one of the best alternatives because of its low cost, longer fiber than straw, low refining energy consumption, good sheet formation [38-40]. At present only 10 million tonnes of sugarcane bagasse is available as surplus for the pulp and paper industry out of 50 million tonnes of bagasse generated in the country per annum. It is difficult to increase the availability of this surplus bagasse to pulp and paper due to present operating circumstances of the sugar industry [41]. Wheat straw is also abundantly available agricultural residue, which is utilized for paper making in India. Approximately, 125 and 170 million tonnes of wheat straw are produced every year in North America and Europe respectively and India produces 131 million tonnes of wheat straw annually. Utilization of wheat straw for paper production is increasing in developing countries such as India and China where there is a lack of good quality of wood and bamboo [42, 43].

Eulaliopsis binata is a perennial grass belonging to family Gramineae which is widely distributed in India and Southern and Central China abundantly. In India, *Eulaliopsis binata* is distributed in Uttar Pradesh, Uttrakhand, Bihar, Madhya Pradesh, Haryana, Punjab and Himachal Pradesh [44, 45]. *Eulaliopsis binata* (Sabai grass) is an excellent raw material for pulp and paper manufacture due to its some unique properties. *Eulaliopsis binata* shows open and loose anatomical structure with lower lignin content which makes it suitable for easy extraction of pulp using milder pulping conditions. *Eulaliopsis binata* fibers are longer that have good strength properties and toughness [44, 46]

There are some advantages in papermaking from non-wood fibres compared to woody plants. The cellulose content in most of the non-wood fibres is comparable to woods that are commonly used for papermaking. The lignin content in non-wood fibres is comparatively low which reduces the chemical consumption and energy during pulping for papermaking [3, 35, 47]. The bleaching of pulp produced from non-wood fibre sources is easier than wood pulp and it also requires lower chemical consumptions with short bleaching sequences [47]. Non-wood

fibres are produced at short growth cycle and they require the moderate irrigation and fertilization during cultivation. The constant availability of substrate throughout the year is primary concern for paper industry. Most of the non-wood fibre comes from annual plants therefore, a storage capacity should sufficiently large which may ensure proper supply of raw materials. Furthermore, most of the non-wood fibre are low density materials and have higher volume compared to wood which makes the situation more complicated [3, 35]. High silica content in non-wood plant fibres is a problem associated with chemical recovery (Table 1.4). During cooking, silica is dissolved, enters the black liquor and creates several problems in chemical recovery. Generally, the number of mills using non-wood fibre is small and they don't have proper chemical recovery system to handle large amount of silica [3, 47].

The pulp and paper industry is one of the most polluting industries and comes under red category of industries [48]. Due to stringent rules of the government, it faces a constant pressure to reduce and modify environmental emissions to air and water. In the process of paper making, chemical pulping of raw material is the first step in which fibers are broken apart and most of the lignin and hemicelluloses are removed. After chemical pulping, residual lignin is removed by the process of bleaching having several stages [49]. Residual lignin after pulping is removed during chlorination stage. Chlorine is added in different forms as molecular chlorine during chlorination stage and hypochlorite during hypochlorite stage. In developing countries, elemental chlorine is used in the majority of the mills while it is banned in developed countries. In developed world, chlorine dioxide, oxygen, ozone, and hydrogen peroxide are used as bleaching chemicals. Elemental chlorine reacts with lignin and other organic compounds in pulp and forms chlorinated compound which are extracted with alkali [49]. Higher chlorine demand during pulp bleaching improves the brightness of pulp but the increase in total chlorine demand adversely affects the strength properties of pulp, stability of brightness, and pollution load. Some of the pollutants such as tannins, resin acid, and stilbenes are the part of extractives present in raw materials. Other compounds are xenobiotic compounds that are generated during different processes like pulping and bleaching of pulp [49]. These compounds include chlorinated lignins, dioxins, furans, resin acids and phenol. Chemical pulp bleaching has become an issue of great concern due to release of adsorable organic halides (AOX). AOX contains more than 300 different organochlorines and some of them are toxic, mutagenic, persistent and bio-accumulating due to their lipophilic nature which

cause numerous harmful disturbances in biological systems [50, 51]. Some chlorinated compounds such as are dioxins, furans are able to induce genetic changes in exposed organisms [49]. C-stage during pulp bleaching is the first point where 2,3,7,8- TCDD, 2,3,7,8-TCDF and 1,2,7,8-TCDF congeners are always present [52-54]. The chlorinated organic compounds generated during the chemical bleaching of pulp have attracted most attention in the recent years. Several absorbable organic halides (AOX) are generated by chlorine-based bleaching and many of them are harmful to health or the environment [55, 56]. Animal carcinogens such as dioxins and furans are produced in chlorination stage of bleaching. Use of such chemically treated paper for different purposes such as baby diapers and packaging of edible products like bread and biscuits, sweetmeat and crystallized fruits, and tea bags is of great concern [55]. The filtrate from E-stage contains the highest concentrations of dioxins which is well known for changing the blood chemistry and causing liver damage, skin disorders, lung lesions and tumor types at numerous sites within the body, including liver and thyroid [52, 57, 58]. The industry is hence looked upon as a notorious pollution maker and unfortunately the truth can't be denied.

The environmental protection regulations were made strict through the world to limit the effluent discharge in environment. Due to growing environmental concerns and legislative pressures on pulp and paper industry is forced to modify its current pulping, bleaching and effluent treatment technologies. To reduce the pollution load, the remedial action can classified in to two approaches that are preventive strategy and curative strategy. In preventive measures cleaner technologies are adopted to reduce the emission of toxic substances during different processes of papermaking. The effluent treatment technologies come under curative methods [49]. It is better to avoid the generation of pollutants compared to perform the treatment of effluent.

The pulping process plays a key role in the formation of pollutants during paper making. During pulping, the removal of lignin as much as possible, reduce the residual lignin in unbleached pulp which can significantly reduce the volume of bleaching chemicals. The targeted brightness can be achieved with lower bleaching chemicals cost and effluent treatment cost. Therefore, various processes were applied to achieve maximum delignification without pulp yield losses during pulping [49, 59-62]. Different methods were utilized to improve the degree of delignification. The degree of delignification improves by extended cooking method

and volume of bleaching chemicals may reduce up to 35% [49, 63, 64]. In oxygen delignification elemental O_2, sodium hydroxide and magnesium hydroxide is used under pressure and it is an expensive method. Oxygen delignification can result reduction in residual lignin up to 50% [59, 65, 66]. Ozone has been proved as efficient competitive bleaching chemical in terms of delignification ability, cost and environmental impact. It has been utilized successfully for variety of raw materials from woods and non-woods [67, 68]. The pretreatment of raw material with microorganisms or enzyme prior to pulping is defined as bio-pulping. Lignin degrading white-rot fungi or enzymes such as ligninases and xylanases have been used for bio-pulping which removes lignin and hemicelluloses. Bio-pulping reduces the consumption of chemical during chemical pulping and it can also reduce the mechanical energy up to 30% during subsequent mechanical pulping. Bio-pulping shows superior physical strength properties compared to conventional methods [69-72]. Organosolve pulping has also been utilized to decrease the residual lignin before bleaching. The use of organic solvent during pulping is known as organosolve pulping. Organic solvents are used either alone or in combination with soda or kraft cooking chemicals. The solvent increases the selectivity of alkali towards lignin and improves the pulp yield and strength properties significantly along with reduced residual lignin [73-75]. Many researchers have used different methods for the reduction of kappa number in kraft pulp in order to reduce the bleaching chemical consumption. These methods include, high sulphidity cooking, leveling out alkali profile throughout the cook, using wood chips from trees of suitable age and temperature decrease throughout the cooking cycle [76].

Residual lignin in pulp imparts dark colour and it is removed by several stages of bleaching. In the conventional method of bleaching chlorine is used which generates the AOX which makes effluent discharge highly toxic. The focus is on the alternatives of conventional bleaching technologies which can reduce the AOX and total organic chlorides (TOCl) in effluent discharge. Organic chlorides in bleach plant effluents can be mitigated by modifying the conventional bleaching processes. Therefore, several cleaner bleaching sequences are used such as use of cooking additives like anthraquinone and surfactant [77], elemental chlorine free (ECF), total chlorine free (TCF), and enzymatic bleaching [49, 78]. ECF bleaching uses the chlorine dioxide in place of chlorine, resulting lower AOX in effluent discharge with acceptable quality of high brightness of pulp [79]. Bleaching without elemental chlorine or

chlorine containing compounds is termed as totally chlorine free (TCF) bleaching process. These bleach techniques exploit the oxidative agents such as oxygen, ozone and peroxide, etc. which degrade lignin by oxidation, thereby decreasing the molecular size and increasing its water and alkali solubility [80]. These alternatives are, however, quite expensive to adopt since, they require a lot of changes in the infrastructure and hence, are only viable to large paper mills [81]. For agro residue pulp mills, with production capacity less than 100 tonnes per day, these alternate bleaching technologies may not be feasible on techno-economical grounds in developing countries like India [82]. Moreover, the risk of loss of pulp viscosity and strength is always there [83]. Therefore, switching over to biobleaching has proven to be the most promising alternative for eliminating chlorine based chemicals in the pulp bleaching [78].

Enzymes can serve as a promising biotechnological tool being the best alternative to polluting chemical technologies. Various enzymes such as cellulases, xylanases, lipases, and amylases etc. are used in different processes in pulp and paper industry [84-86]. Xylanases are gaining popularity as a catabolic agent for delignification in the bleaching process [87-91]. The use of enzymes in bleaching is known as biobleaching. The positive effect of xylanases on delignification is attributed due to removal of xylan by breaking the link between cellulose and lignin. During subsequent bleaching stages lignin is eliminated effectively with bleaching chemicals. It is well established that xylan re-deposit on the surface of fibre and acts as physical barrier for bleaching chemicals. Re-deposited xylan increases the chemical consumption during bleaching and it also entraps the lignin which affects the fibre swelling [78, 92, 93]. Therefore, the elimination of the re-deposited xylans by xylanases facilitates the penetration of bleaching chemicals. Xylanases are also commonly used for biobleaching of non-wood pulps [55, 78].

The microbial producers of xylanase are bacteria, actinomycetes and fungi. Enzyme production can be achieved through fermentation techniques such as solid-state fermentation (SSF) and submerged state fermentation (Smf). SSF showed several technical benefits with high product yield [94, 95].

The major objectives of the thesis are as under:

1. Isolation, screening and selection of xylanase producing fungi with minimum cellulase activity.

2. To enhance xylanase production by optimizing the cultural parameters and partial purification and characterization of xylanases from selected fungal isolates.

3. Morphological and proximate chemical studies of *Eulaliopsis binata* to check its suitability and potential for production of chemical grade pulps.

3. Optimization of soda pulping process in order to reduce the kappa number prior to bleaching and escalate the pulp yield of *Eulaliopsis binata* and effect of ethanol-soda and bio-soda pulping on pulp yield, optical and strength properties of *Eulaliopsis binata* pulp.

4. To enhance optical and strength properties of *Eulaliopsis binata* pulp by using fungal xylanase during bleaching experiments and to reduce the toxicity of effluents generated during conventional, ECF and TCF bleaching sequences of the respective pulps to make the process eco-friendly and cost effective.

Table-1.1: Growth of per capita consumption and paper mills in India [9]

Year	No of units	Installed capacity (million tonnes)	Production (million tonnes)	Capacity utilization (%)	Per capita consumption (kgs)
1950	17	0.13	0.11	85	0.9
1970	57	0.77	0.75	99	1.9
1990	325	3.3	2.43	62	3.6
2000	380	3.94	4.87	99	5.5
2006	660	8.5	6.8	80	6.7
2007	667	8.5	8.3	100	8.3
2010	759	12.7	10.11	80	9.3

Table-1.2: Gross crop residue biomass potential in India [96]

Crop group	**Crop**	**Gross potential (MT)**
Cereals	Bajra	24.3
	Barley	1.6
	Jowar	17.6
	Maize	35.8
	Ragi	2.7
	Rice	154.0
	Small millet	0.6
	Wheat	131.1
Oilseeds	Groundnut	17.0
	Linseed	0.3
	Mustard and rapeseed	12.7
	Niger	0.1
	Saflower	0.6
	Sesame	0.8
	Soybean	13.5
	Sunflower	3.8
Pulses	Gaur	2.6
	Gram	6.4
	Lentil	1.7
	Tur (arhar)	7.2
Sugarcane	Sugarcane	110.6
Horticulture	Arecanut	1.5
	Banana	41.9
	Coconut	18.0
Others	Cotton	75.9
	Jute	3.9
Total (MT)		686.0

Table-1.3: Average annual yields of different papermaking raw materials [97]

S. No.	Plant	Fibre yield (tonnes/year/ha)	Pulp yield (tonnes/year/ha)
1	Bagasse	9	4.2
2	Bamboo	4	1.6
3	Canary grass	8	4.0
4	Elephant grass	12	5.7
5	Fast growing hardwood	15.	7.4
6	Fast growing softwood	8.6	4.0
7	Hemp	15	6.7
8	Kenaf	15	6.5
9	Rice straw	3	1.2
10	Scandinavian softwood	1.5	0.7
11	Temperate softwood	3.4	1.7
12	Wheat straw	4.	1.9

Table-1.4: Chemical compositions of different papermaking raw materials [71, 98]

Lignocellulosic materials	α-Cellulose (%)	Lignin (%)	Inorganic elements (%)	Silica (%)
Hardwoods	38-48	23-30	0-1	0
Kenaf	31-39	14-19	2-5	NA
Maize stalk	NA	22-24	5-6	3-5
Oats straw	31-37	16-19	6-8	4-7
Oil palm frond	49.8	20.5	-	-
Rice straw	28-36	12-16	15-20	9-14
Rice husk	38-40	22-24	20-22	19-20
Softwoods	40-45	26-34	0-1	0
Sugarcane bagasse	32-44	19-24	2-5	3-7
Wheat straw	38-46	16-21	5-9	3-7

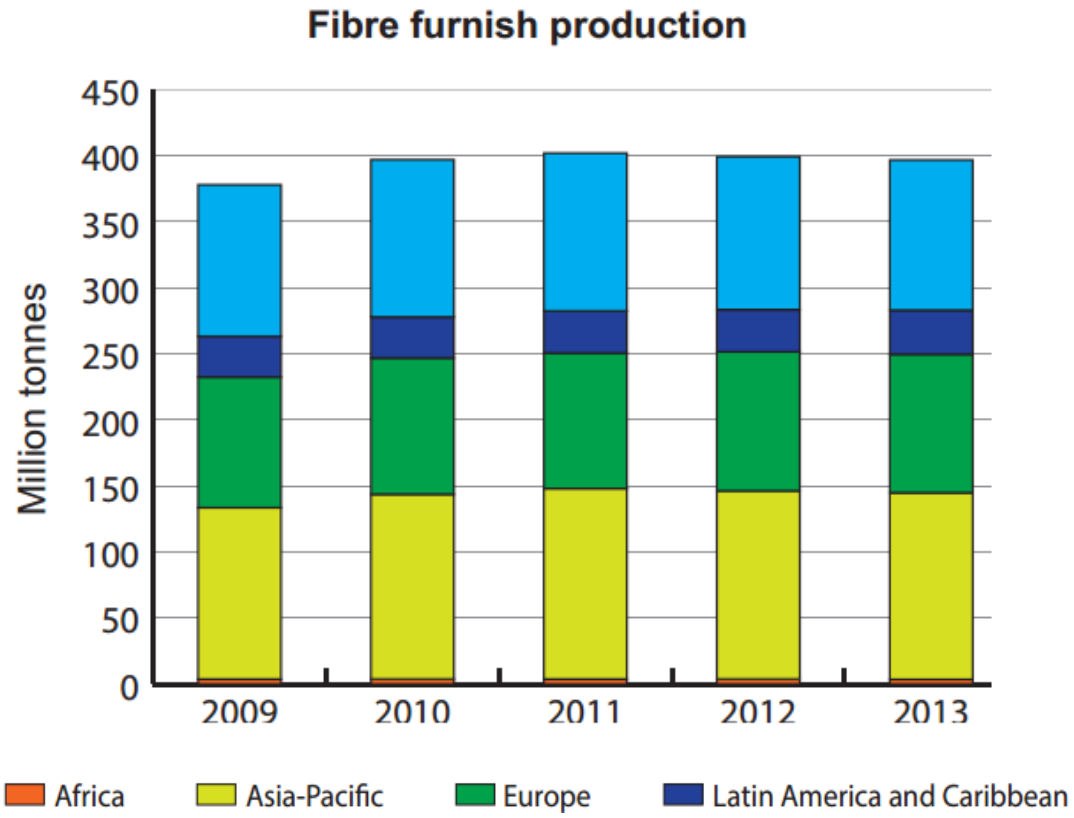

Figure-1.1: Global fibre furnish production (2009-2013) [2]

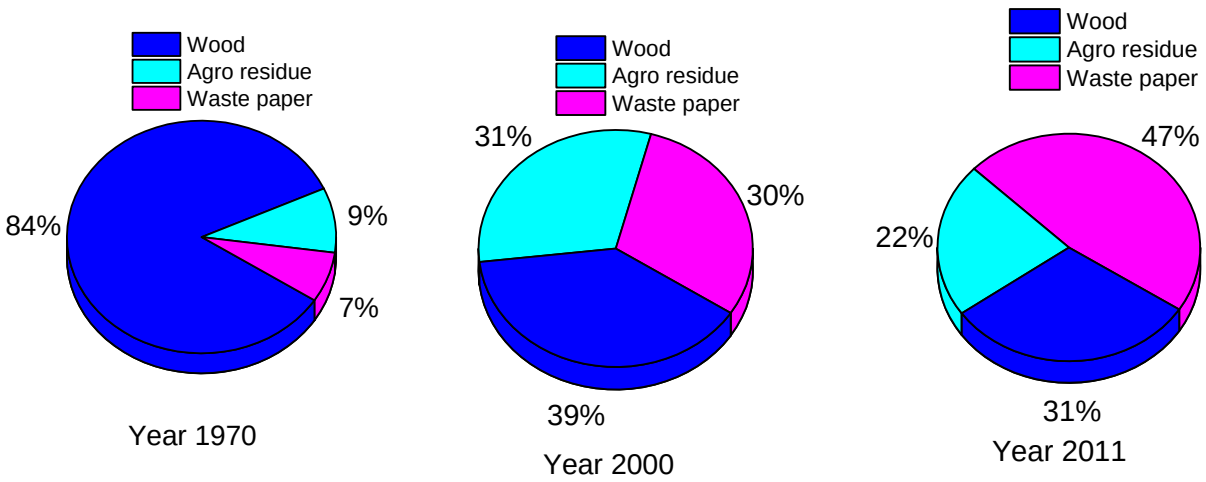

Figure 1.2 Major raw materials for paper, paperboard and newsprint in India [5]

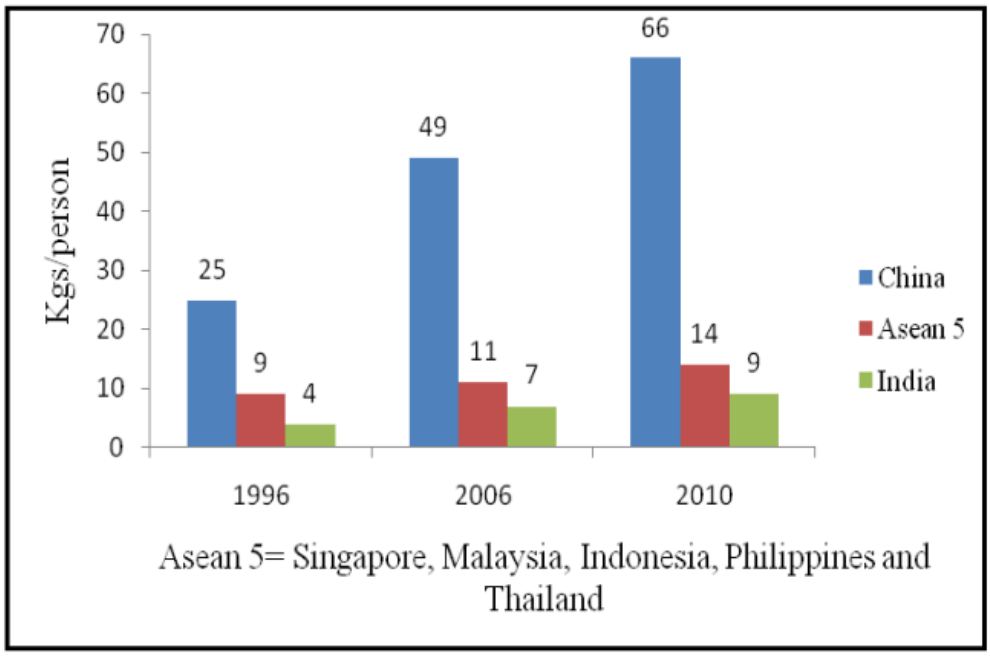

Figure-1.3: Per capita consumption of paper and paperboard in Asia [99]

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CHAPTER 2

XYLANASE PRODUCTION

2.1: Introduction

Biocatalysis offers the green and clean solution to chemical processes and it is emerging as a challenging and revered alternative to chemical technology. Enzymes are the proteins which considered as potential biocatalysts for a large number of reactions. In living system, enzymes function in transformation of macromolecules to energy and new materials, besides for their growth, repair and maintenance of cells. Enzymes are derived from plants, animals and microorganisms, but microorganisms are preferred for commercial production of enzymes due to ease of growth, nutritional requirement and downstream processing. Furthermore, microbes produce higher quantity of enzymes in comparison to plants and animals sources. Most of the microbial enzymes are inducible and therefore, their production can be enhanced significantly with the addition of inducers in the production media [1, 2].

Xylanases are hydrolases which depolymerize the xylan and is the second most abundantly available polysaccharide [3]. Xylanases are synthesized by many microorganisms, like fungi, bacteria and actinomycetes. However, fungi are the most interesting sources of xylanases due to higher yield of extracellular xylanase production. The extracellular xylanases act on hemicelluloses to release xylose which is assimilated by organisms to grow heterotrophically on xylan [4-6]. Extracellular enzymes are advantageous because of their easy extraction procedure [7]. Ascomycetes have been studied most extensively for xylanases production. The different species from three genera namely *Trichoderma, Aspergilli and Penicillia* dominates in literature for xylanase production [8]. Several species of genus *Aspergillus* including *A. niger, A. oryzae, A. fumigatus, A. terreus, A. awamori, A. nidulans* have been extensively studied for xylanases [9, 10]. Different species of *Tricoderma* has been widely studied for xylanase production. *Trichoderma* species such as *Trichoderma reesei, T. viride, T. harzianum, T. lignorum, T. longibrachiatum, T. koningii and T. pseudokoningii* have been reported for xylanase production [11]. Different species of genus *Penicillium* such as *P. brasilianum, P. chrysogenum, P. citrinum, P. oxalicum, P. notatum, P. chrysogenum, P. funiculosum, P. hirsutum, P. janthinellum, P. pinophilum, P. verruculosum* etc. have been

studied for xylanase production [8, 12, 13]. Along with these fungi some other fungi such as *Chaetomium, Fusarium, Humicola, Talaromyces*, and many others have been proved effective for xylanase production [7, 14, 15].

Hemicelluloses are branched heteropolymer which consists of pentoses (D-xylose and D-arabinose) and hexoses (D-mannose, D-glucose and D-galactose). The biodegradation of hemicelluloses requires a set of esterases and glycanases. These enzymes act synergistically on hemicelluloses for its complete hydrolysis [16, 17]. These enzymes are:

- Endo-1,4-β-xylanases (E.C. 3.2.1.8.) randomly act on homopolymeric xylan chain to produce the mixture of xylooligosacchaides.
- β-Xylosidases (E.C. 3.2.1.37.) release xylose from non-reducing end of small oligosaccharides or xylobiose.
- α-L-Arabinofuranosidases (E.C. 3.2.1.55.) acts on L-arabinofuranose side chains.
- α-D-Glucuronidases (E.C. 3.2.1.139.) hydrolyze methyl glucuronate residue.
- Acetyl xylan esterase (3.1.1.72) removes acetate groups from main chain.
- Feruloyl (E.C. 3.1.1.73) and coumaryl esterases act on respective aromatic acids to arabinofuranoside residues.

Among these enzymes endo-1,4-β-xylanase and β- xsylosidases (collectively xylanases) are the two main enzymes which are responsible for the xylan hydrolysis [8, 18-20]. Xylanases have wide biotechnological applications such as saccharification of lignocellulosic biomass [21, 22], improving digestibility of animal feed [23], and clarification of wines and fruit juices [24, 25], desizing of cotton and micropoly fabrics in textile industry and improving bakery products [4, 24]. Xylanases are the dominating enzymes in pulp and paper industry due to their several applications. Xylanases have been utilized in pulp and paper industry for different process such as pre-bleaching of pulp for reducing the consumption of bleaching chemicals [26, 27], deinking of waste paper [28-30], and energy reduction and improving hydrogen bonding during refining and improvement in drainage at wet-end part of paper machine [31, 32]. Xylanases hydrolyze the relocated and precipitated hemicelluloses on the surface of cellulosic fibre which increases the permeability for oxidizing agents by attacking the lignin-carbohydrate complexes [10]. Few xylanases preparations have contamination of cellulases which can adversely affect the strength of pulp. Therefore, cellulase-free xylanases are required for the pre-bleaching of pulp.

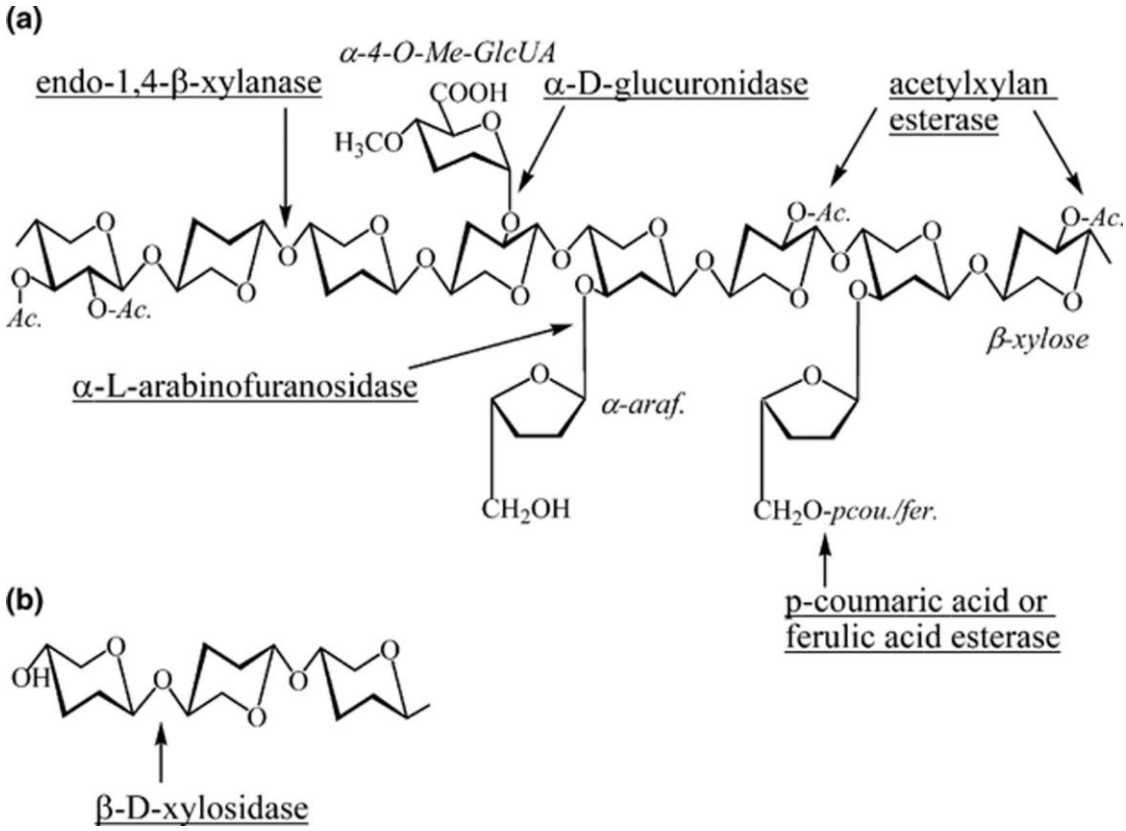

Figure 2.1: Site of action for different xylanases [20]

The major limitation for industrial applications of xylanases is their production cost. The use of purified xylan as substrate makes the xylanase production costly. Therefore, simple and inexpensive carbon sources are required for the production of xylanase at commercial scale [7]. The utilization of abundantly available and low cost agricultural residue as the carbon sources can be employed to decrease the xylanase production cost. Wheat bran, wheat straw, corn cob, rice straw, rice husk, sugarcane bagasse etc. have been reported as efficient substrates for xylanase production [7, 33]. Production of xylanase has been studied in both solid-state fermentation (SSF) and submerged fermentation (SmF) conditions. Further, cost reduction for xylanase production can be attained by using SSF technique for xylanase production. SSF involves the growth of microorganism on a wet solid substrate in absence or near absence of free water. SSF gains interest due to a number economic and engineering advantages including simplicity of equipment, low energy consumption and the lower moisture content which prevents the bacterial contamination [34-36]. The advantages of SSF compared to traditional

SmF are higher yields, easier products recovery and use of lesser amount of solvent for enzyme extraction which reduces the downstream processing cost significantly [34-38].

Present work aims at isolation, screening and selection of xylanase producing fungi from the wood decaying samples, decomposing manure and soil samples from diverse habitat. Different agro-residues were tested for the production of xylanolytic enzymes by selected fungal strains under solid-state fermentation. Optimization of various cultural physiochemical parameters was carried out to achieve maximum enzyme production from the screened fungal strains. Biochemical characterization of xylanases was carried out to check their temperature and pH optima for their successful utilization in pre-bleaching studies.

2.2: Materials and methods

2.2.1: Materials

Chemical used throughout the study were of analytical grade. Sodium salt of carboxymethylcellulose (CMC) of medium viscosity and birch wood xylan were procured from Sigma Chemical Co. St Louis, MO, USA. Ammonium chloride, diammonium phosphate, ammonium sulphate, sodium nitrate, urea, D-glucose, D-xylose, di-nitrosalisylic acid (DNS), sodium nitrate, potassium sodium tartarate, p-nitro phenyl-β-D glucopyranoside (p-NPG), p-nitrophenol, Tween-80, Tween-60, Tween-40, Tween-20, Triton-X-100, and bovine serum albumin (BSA) were purchased from HiMedia Chemicals (India). Ammonium nitrate was purchased from Qualigens Fine Chemicals Pvt., Ltd., India. Whatman filter paper no.1 was purchased from GE Whatman. Agar-agar, beef extract, malt extract, peptone, potato carrot agar, tryptone, yeast extract, and other media ingredients were purchased from HiMedia Biosciences, India. Soya bean meal (defatted) was purchased from Loba Chemie, Laboratory reagents and fine Chemicals, India.

2.2.2: Isolation of xylan degrading fungi

The various samples containing microbes were collected from different locations of three states namely Uttar Pradesh, Uttarakhand and Rajasthan situated at northern part of India. Different samples of decomposing manures, dead and decaying wood and soil enriched with lignocellulose were collected using sterilized spatula and polythene bags. Isolation of fungal isolates was carried out using dilution plate method. One gram of each sample was homogenized manually and transferred to test tube containing ten milliliter of sterilized

distilled water. It was shaken vigorously and the suspension was subjected to serial dilution up to 10^{-7}. After serial dilution, plating was carried out from dilutions ranging from 10^{-4} to 10^{-7}. Primary screening for xylanase producing fungi was carried out on xylan agar medium composed of various constituents expressed as g/l: 5.0 xylan, 5.0 peptone, 5.0 NaCl, 3.0 yeast extract and 15.0 agar. Chloramphenicol (20 µg/ml) and Rose Bengal (30 ppm) were also added to medium to prevent bacterial growth [18]. Petri-plates were incubated at 30 ºC and examined after 2-3 days regularly and growing colonies were selected for further xylanase detection. The selected colonies were isolated, purified on potato dextrose agar (PDA) and maintained over PDA slants at 4 ºC.

2.2.3: Screening for xylanase producing fungi

Isolated fungal strains were analyzed for zone assay and potential xylanase producer were selected. Zone assay performed on xylan-agar medium, composed of various constituents expressed as g/l: 5.0 xylan, 5.0 peptone, 5.0 yeast extract, 1.0 K_2HPO_4, 0.2 $MgSO_4.7H_2O$ and 15.0 agar [39]. For zone assay xylan-agar plates were flooded with an aqueous solution of Congo red consisting of 0.5% Congo red and 5% (v/v) ethanol in distilled water and incubated for 15 min. To enhance the visibility of clear zone the excess of dye was removed by destaining with 1 M NaCl. [51].

2.2.4: Xylanase production under solid-state fermentation

Xylanase production was carried out under solid-state fermentation (SSF) using wheat bran as the substrate. Wheat bran (5 g) was moistened with Mandel Weber medium (77.5 % initial moisture content) with following composition expressed as g/l: 1.4 $(NH4)_2SO_4$, 2.0 $KH2PO_4$, 0.3 $CaCl_2$, 0.3 $MgSO_4.7H_2O$, 0.02 Tween-80 and trace elements: 0.005 $FeSO_4.7H_2O$, 0.0016 $MnSO_4.7H_2O$, 0.0014 $ZnSO_4.7H_2O$, 0.002 $CoCl_2.6H_2O$. The initial pH of Mandel Weber medium for enzyme production was maintained to 5.5 with 1.0 N HCl or 1.0 N NaOH. Flasks were inoculated with 10^6 spores/gds or five discs of actively growing fungi for spores producing and spores lacking fungi respectively and incubated at 30 ºC for 6 days [37, 40, 41]. For enzyme extraction 50 ml of distilled water was added the fermented wheat bran and shaken for 60 min at 150 rpm and 30 °C temperature in an rotator incubator shaker.

2.2.5: Enzyme assays

Xylanase activity was determined by estimating the reducing sugars released by 1% (w/v) of birch wood xylan (Sigma Chemical Co. St Louis, MO, USA) in 50 mM citrate buffer at pH 5.5 according to Bailey method [42].]. Xylanase activity was determined by incubating the 1.6 ml of xylan solution with 0.4 ml of appropriately diluted enzyme at 50 ºC for 15 min. Quantification of reducing sugars was done at 540 nm using UV-Vis spectrophotometer (SHIMADZU, UV-1800) by dinitrosalicylic acid (DNS) method [43]. One unit of xylanase activity is defined as the amount of enzyme that releases 1 µmole of xylose per min per ml under reaction conditions. Xylanase activity was expressed as activity units per mass of initial dry solid substrates (IU/gds).

Cellulase activity was determined by standard method recommended by International Union of Pure and Applied Chemistry (IUPAC). Cellulase activity was determined by incubating 0.5 ml of 2% (w/v) carboxymethyl cellulose of medium viscosity (Sigma Chemical Co. St Louis, MO, USA) with 0.5 ml of appropriately diluted crude enzyme for 30 min [44]. Cellulase activity was determined at temperature 50 ºC using citrate buffer of 50mM and pH 5.5. Quantification of reducing sugars was done at 540 nm using UV-Vis spectrophotometer (SHIMADZU, UV-1800) by dinitrosalicylic acid (DNS) method [43]. One unit of cellulase activity is defined as the amount of enzyme required to liberate 1 µmole of glucose per min per ml under reaction conditions.

Laccase activity was estimated using ABTS (2, 2'-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid) as the substrate [45]. In the reaction mixture 0.6 mL of 100 mM citric acid buffer of pH 5.5 was taken and 1.0 mL of enzyme extract, 0.2 mL of 1.0 mM ABTS and 0.2 mL of distilled water were added. The reaction was monitored using UV-Vis spectrophotometer (SHIMADZU, UV-1800) at 420 nm at room temperature. Enzyme activity was expressed as the amount of enzyme, which produced an increase of 1.0 absorbance unit per 30 s.

2.2.6: Estimation of protein concentration

Protein content was determined using bovine serum albumin (BSA) as the standard according to the Lowry method as described below:

Reagents

Reagent-A: 2 % Na_2CO_3 in 0.1 N NaOH

Reagent-B: 1 % $CuSO_4.5H_2O$ in distilled water

Reagent-C: 2 % Sodium potassium tartarate in distilled water

Lowry solution- Reagents A+B+C in the ratio of 100:1:1

200 µl of protein sample was added into 2 ml of Lowry solution and mixed well. After that, it was allowed to stand for 10 min at room temperature. After 10 min, 200 µl of Folin–Ciocalteu′s phenol reagent (1 N) was added and incubated for 30 min at room temperature. Absorbance of sample was taken at 550 nm with UV-Vis spectrophotometer (SHIMADZU, UV-1800). The protein content of samples was determined by comparing the absorbance of protein samples with that of a standard curve of bovine serum albumin [46, 47].

2.2.7: Identification of selected fungal strains

The fungal isolates ARC-11 and ARC-12 were selected for xylanase production. Both the isolates were sent to National Fungal Culture Collection of India, Agharkar Research Institute, Pune for ITS sequencing and identification. ITS1-5.8S-ITS2 sequencing of both the fungal strains was carried out for phylogenetic analysis and morphological features were also analyzed for both the fungal strains.

2.2.8: FE-SEM analysis of selected fungal strains

Morphological features of both the fungal strains were analyzed using FE-SEM (MIRA3 TESCAN). Fungal mat was treated with 3% glutaraldehyde (v/v) and 2% formaldehyde (4:1) for 6 h for fixation. The samples were washed thrice with distilled water after fixation and then treated with ethanol gradients of 30-90% with a difference of 10% for 15 min. In the next step of dehydration, samples were treated with absolute ethyl alcohol (99.9%) for 30 min. After dehydration, samples were air-dried and coated with gold by a standard sputtering technique for 30 s. Electron photomicrographs were taken at suitable voltage and magnifications.

2.2.9: Agro-residues as the substrate for enzyme production

For xylanase production different agro-residues such as corn cob, corn stover, congress grass, maize bran, pearl millet stover, rice straw, rice husk, sabai grass, sugarcane bagasse, sugarcane tops, sun hemp residue, wheat bran and wheat straw were collected from Muzaffarnagar district in Uttar Pradesh, India. All the agro-residues were washed to remove dirt particles and dried in sunlight. The agro-residues were chopped in to 1-2 cm pieces by using fodder cutter machine. After chopping, all the residues ground in a Wiley mill in the particle size range of 250 to 1400 µm and they were used as the substrates for xylanase production. To select the suitable carbon source for xylanase production these ago-residues were utilized as the substrate under SSF conditions.

2.2.10: Optimization of physiochemical parameters for xylanase production

Effect of different physiochemical parameters on xylanase production was tested by using one factor at a time (OFAT) approach [37, 48]. For the selection of best carbon source, eleven different agro-residues were utilized for xylanase production under SSF conditions.

2.2.10.1: Effect of incubation time

To optimize incubation time for xylanase production, Erlenmeyer flasks (250 ml) containing five gram of suitable substrate were prepared. Xylanase was harvested up to 96 h with a gap of 12 h for fungal strain ARC-12 while for fungal strain ARC-11 xylanase was harvested up to 12 days with a difference of one day. Initial pH and moisture content were adjusted to 5.5 and 77.5% respectively and incubated at 30 °C. The estimation of xylanase activity and protein content was carried out as per methods described in subsection 2.2.5.

2.2.10.2: Effect of temperature

The effect of incubation temperature on xylanase production was studied by incubating the inoculated flasks at different temperatures (26-42 ºC) at an interval of 4 ºC. Enzyme harvesting was carried out at an optimum incubation time as determined previously while keeping the other fermentation conditions constant as described above.

2.2.10.3: Effect of initial pH

For optimization of pH, xylanase production was carried out an optimum incubation time and temperature as determined previously. The effect of initial pH on enzyme production was assessed by adjusting the initial pH of production medium at 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 9.0 and 10.0 with 1.0 N HCl or 1.0 N NaOH.

2.2.10.4: Effect of moisture content

The effect of moisture content on xylanase production was assessed by varying the initial moisture contents i.e. 55.0, 65.0, 70.0, 75.0, 77.5, 80.0, 82.5, and 85 % while maintaining the nutrients concentration constant at optimum cultural conditions. The estimation of xylanase activity and protein content was carried out as described in section 2.2.5.

2.2.10.5: Effect of nitrogen sources

The influence of nitrogen sources on xylanase production was studied at optimum cultural conditions such as incubation time (8^{th} day), temperature (30 ºC), initial pH (7.0) and initial moisture content (70.0%) for fungal strain ARC-11. For fungal strain ARC-12, influence of nitrogen sources on xylanase production was studied at optimum cultural conditions such incubation time (48 h), temperature (30 ºC), initial pH (6.0) and initial moisture content (77.5%). The effect of nitrogen sources on enzyme production was studied by replacing $(NH_4)_2SO_4$ in Mandel Weber medium with different nitrogen sources such as NH_4Cl, NH_4NO_3, $(NH_4)_2PO_4$, $(NH_4)_2SO_4$, $NaNO_3$, HN_2CONH_2 (simple nitrogen sources) and beef extract, malt extract, peptone, soybean meal, tryptone, and yeast extract (complex nitrogen sources). All the nitrogen sources were tested at four different concentration levels i.e. 0.04, 0.08, 0.12, and 0.16 % (as available nitrogen basis) for simple nitrogen sources while the complex organic nitrogen sources were supplemented as 0.4, 0.8, 1.2, and 1.6 % (as w/v basis).

2.2.10.6: Effect of surfactants

In order to observe the effect of surfactant on enzyme production, various surfactants (Tween-20, Tween-40, Tween-60, Tween-80, Triton-x-100, SDS and EDTA) were added at concentrations like 0.05, 0.10, and 0.20 and 0.30 % (w/v) to the NSS solution.

2.2.11: Bio-chemical characterization of crude xylanase

2.2.11.1: Partial purification of xylanase by ammonium sulphate precipitation

The cell free supernatant of crude extract was subjected to fractional ammonium sulphate precipitation. Finely powdered AR-grade ammonium sulphate was added to 200 ml of crude extract at different saturation levels of 10% to 100% w/v. Ammonium sulphate was added slowly to crude enzyme with continuous stirring at 4 °C and incubated overnight at 4 °C. The precipitate was collected after centrifugation at 9,000x g for 30 min and dissolved in minimal amount of citrate buffer (concentration 50 mM) of pH 5.5. Protein content and xylanase activities were determined for each fraction. The fractions which showed significantly higher xylanase activities were pooled together and dialyzed to remove ammonium sulphate from xylanase solution. Dialysis was performed against the same buffer (pH 5.5 and 50 mM) with dialysis membrane at 6 °C for 24 h. During dialysis process buffer was changed frequently till no more ammonium sulphate was detected. The undissolved dialysate in xylanase enzyme solution was removed after centrifugation at 9,000x g for 20 min at 4 °C.

2.2.11.2: Optimum pH and temperature

Optimum pH for xylanase activities from fungal strains ARC-11 and ARC-12 were determined by assaying their activities at different pH (3.0 to 9.0) and fixed temperature i.e. 50 °C. Different buffers at fixed concentration (50 mM) such as sodium citrate buffer for a pH range of 3.0 to 6.0, sodium phosphate buffer for a pH range of 6.5 to 8.0 and glycine-NaOH buffer for a pH of 9.0 were used to maintain desired pH. Similarly, xylanase assays were determined at different reaction temperatures varying from 40 to 75 °C with difference of 5 °C using sodium citrate buffer maintaining their respective pH at 5.0 and 6.0 for both the fungal strains i.e. ARC-11 and ARC-12 respectively.

2.2.11.3: Thermo-stability of xylanases

Thermo-stabilities of xylanases from fungal strains ARC-11 and ARC-12 were determined at temperature 45, 50, 55, and 60 °C. Thermo-stabilities of xylanases were estimated by pre-incubating the enzymes at different holding times ranging from 0 to 180 min at optimum pH. After holding the enzyme preparation at different temperature and time, xylanase assays were performed at respective optimum temperature and pH given in subsection 2.2.11.2.

2.2.11.4: Effect of cations on partially purified xylanases

The influence of metal ions on xylanase activity was studied at two different concentrations (1 and 10 mM). Different metal ions such as Na^{+}, K^{+}, Al^{+++}, Ca^{++}, Cd^{++}, Co^{++}, Cu^{++}, Fe^{++}, Hg^{++}, Mn^{++}, Mg^{++}, Ni^{++}, Pb^{++}, and Zn^{++} were incubated with xylanase for a period of hour at room temperature and then analyzed for their respective xylanase activities (subsection 2.2.5).The residual xylanase activities were measured in presence of salt of each ion. Xylanase activity in absence of metal ions (control) was taken as 100%.

2.2.12: Statistical analysis

All the experiments were carried out in triplicate independently and experimental results were represented as the mean ± standard deviation of three identical values.

2.3: Results and discussion

2.3.1. Isolation and screening of fungi

A total of 112 fungal isolates were obtained after primary screening and showed growth on xylan-agar medium. After plate assay (secondary screening), 20 fungal isolates were selected as potent xylanase producer. Xylanase and cellulase activities were determined for all the fungal strains. Out of 20, only two isolates namely ARC-11 and ARC-12 were selected based on maximum xylanase and minimal cellulase activities (Table 2.1).

2.3.2. Identification of selected fungal isolates

ITS1-5.8S-ITS2 sequencing of fungal strains ARC-11 and ARC-12 was carried out at National Fungal Culture Collection of India, Agharkar Research Institute, Pune (India). Fungal strains ARC-11 and ARC-12 were identified as *Schizophyllum commune* and *Aspergillus flavus* based on the comparison of ITS rDNA gene sequences and morphological characteristics. Identified fungal strains were designated as *Schizophyllum commune* ARC-11 and *Aspergillus flavus* ARC-12 and deposited with accession numbers NFCCI 3029 and NFCCI 3028 respectively at the same centre.

ITS1-5.8S-ITS2 sequences of *Schizophyllum commune* and *Aspergillus flavus*

>Schizophyllum commune (ARC 11)
CGGTTGACTACGTCTACCTCACACCTTAAAGTATGTTAACGAATGTAATCATGGTCTTGA
CAGACCCTAAAAAGTTAATACAACTTTCGACAACGGATCTCTTGGCTCTCGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT
TTGAACGCACCTTGCGCCCTTTGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCATTAAA
TACCATCAACCCTCTTTTGACTTCGGTCTCGAGAGTGGCTTGGAAGTGGAGGTCTGCTGG
AGCCTAACGGAGCCAGCTCCTCTTAAATGTATTAGCGGATTTCCCTTGCGGGATCGCGTC
TCCGATGTGATAATTTCTACGTCGTTGACCATCTCGGGGCTGACCTAGTCAGTTTCAATA
GGAGTCTGCTTCTAACCGTCTCTTGACCGAGACTAGCGACTTGTGCGCTAACTTTTGACT
TGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATAT-AATAA
>Aspergillus flavus (ARC 12)
TTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCG
AGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGGCCCGTccccccGGAGAGGGGACGAC
GACCCAACACACAAGCCGTGCTTGATGGGCAGCAATGACGCTCGGACAGGCATGCCCCCC
GGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACGGAATTCTGCAATT
CACACTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTG
TTGAAAGTTTTAACTGATTGCGATACAATCAACTCAGACTTCACTAGATCAGACAGAGTT
CGTGGTGTCTCCGGCGGGCGCGGGCCCGGGGCTGAGAGCCCCCGGCGGCCATGAATGGCG
GGCCCGCCGAAGCAACTAAGGTACAGTAAACACGGGTGGGAGGTTGGGCTCGCTAGGAAC
CCTACACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGT

S. commune ARC-11 showed white and floccose colonies on Sabouraud dextrose agar (SDA) medium and showed growth up to 32x32 mm in diameter on 6th day of incubations. The mycelium of the fungal strain *S. commune* ARC-11 contained two types of hyphae that were thick and thin up to 5 µm but sometimes might be coiled. Sclerotia were globose, olivaceous brown and 9-11x5-11 µm in size. *A. flavus* ARC-12 showed colonies on dull herbage green (yellowish green center), sulcate, floccose white on SDA medium. The fungi was fast growing and grown upto 38x38mm diameter on 6th day of incubation. The reverse of plate was yellowish to buff. *A. flavus* ARC-12 showed colourless and roughened conidiophores. Vesicles were globose to sub-globose, flask shaped and hyaline. Sterigmata were uniseriate and biseriate with separate heads. The uniseriate sterigmata were ampulliform, olivaceous to sub-hyaline and 8-12.5x4.5-5 µm in size. The biseriate sterigmata were spatulate. Vesicles were fertile and 17.5x7.5 µm in size. Spores were olivaceous to sub-hyaline, rough walled, globose to oval to sub-globose and measured the size upto 6.5-2.5x5-2.5 µm.

2.3.3. Selection of agro-residue for maximum xylanase production

Different agro-residues such as corn cob, corn stover, congress grass, maize bran, pearl millet stover, rice straw, rice husk, sabai grass, sugarcane bagasse, sugarcane tops, sun hemp residue, wheat bran and wheat straw were used as the carbon sources for xylanase production. The maximum xylanase production from *S. commune* ARC-11 was found with rice straw (4288.36 IU/gds) as the carbon source under SSF conditions (Figure 2.2). *S. commune* ARC-11 was found efficient producer of xylanase which produced fairly high xylanase activity compared to other agro-residues tested for xylanase production under SSF conditions. Agro-residues such as maize bran, wheat straw, sabai grass and sugarcane bagasse gave xylanase yield by 92.33, 87.95, 85.85 and 84.93% respectively compared to rice straw (Table 2.2). Compared to other agro-residues such as congress grass, wheat bran, maize bran, and rice straw, pearl millet stover showed maximum production of xylanase (1345.44 IU/gds) by *A. flavus* ARC-12. Rice straw (1215.03 IU/gds) and maize bran (1216.00 IU/gds) showed the second highest xylanase activity showed with *A. flavus* ARC-12 (Figure 2.3). However, the difference between xylanase activities of *A. flavus* ARC-12 using rice straw and maize bran as the carbon source was insignificant. Rice straw and pearl millet stover was selected as the carbon sources for maximum xylanase production from *S. commune* ARC-11 and *A. flavus* ARC-12 respectively. The use of agro-residues as the source of carbon for xylanase production has been studied in several reports [49, 50]. The cost, availability and physicochemical characteristics of substrates are the main factors during their selection as the substrates for enzyme production [51]. Enzyme production is dependent on the nature of carbon source, favorable degradability, bare chemical composition, physical associations, accessibility of substrate, and presence of some nutrients [52]. Rice straw is rich in hemicelluloses (19-27%) and hence found to be the suitable carbon source for xylanase production [7, 49]. A substrate with higher content of xylan induces the higher xylanase production [50]. During SSF, moisture is absorbed by the substrate particles and microorganisms utilize moisture for their growth and metabolic activities. The degree of hydration of substrate particles affects the microbial growth as well as enzyme production. Water absorption capacity of different substrates varies from one substrate to another. This may be the another possible reason for variation in xylanase production by different substrates as the carbon source during SSF conditions [53, 54]. Xylanase production was carried out using rice straw as the substrate under

SSF by several fungal strains such as *Aspergillus fumigatus* [55], *Fusarium solani* F7 [7], *Trichoderma reesei* Rut C-30 [56], *Myceliophthora* sp. IMI 387099 [57]. Few reports were available on xylanase production by the white-rot fungus, *Schizophyllum commune* [58, 59]. Kolenova et al. [58] observed maximum xylanase activity (71.3 U/ml) by *Schizophyllum commune* under submerged fermentation after 11th day of cultivation on cellulose containing medium. de Souza et al. [60] reported maximum xylanase (190 U/ml) and β-xylosidase (35 U/ml) production by *Aspergillus flavus* using corn cob as the carbon source. Guimaraes et al. [61] found maximum xylanase (8.03 and 8.70 U/mg of protein) production using different concentrations of wheat bran (1 and 0.5%) as the carbon source under submerged fermentation conditions by *Aspergillus flavus*. Several species of genus *Aspergillus* were studied extensively for xylanase production using different agro-residues as the carbon sources. *Aspergillus awamori* [35], *Aspergillus fumigatus* [62], *Aspergillus lentulus* , *Aspergillus niger* [63], and *Aspergillus foetidus* [64] produced maximal xylanase production using different agro-residues as the carbon sources such as grape pomace, rice straw, wheat bran, and corn cob.

2.3.4. Effect of physiochemical parameters for xylanase production

The effect of different physiochemical parameters such as composition of fermentation medium, fermentation duration, pH, incubation temperature, and moisture content during SSF of both the strains to get enhanced production of xylanase was studied.

2.3.4.1: Effect of incubation time on xylanase production

The effect of incubation time on xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12 using rice straw and pearl millet stover as the substrates under SSF has shown in Figures 2.4 & 2.5. The xylanase activity of *S. commune* ARC-11 enhanced gradually with increasing incubation period and reached at the highest level on 8th day of incubation at 30 °C i.e. 5199.02 IU/gds. Further increase in the incubation time i.e. after 8th day, xylanase activity started to decrease (Table 2.3). *A. flavus* ARC-12 was fast growing fungus therefore, xylanase production was monitored after every 12 h. For *A flavus* ARC-12, maximal xylanase (1424.69 IU/gds) production was observed after 48 h of incubation time at 30 °C and beyond that xylanase activity was declined (Table 2.4). The relatively shorter incubation time for maximum production of xylanase minimizes the risk of contamination and makes the process economic for industrial production of xylanase [65, 66]. For *A. flavus* ARC-12, protein concentration

increased from 12 h to 60 h and slightly decreased thereafter. For *S. commune* ARC-11, protein content increased up to 9th day of incubation and beyond that it became almost constant. Enzyme production reaches to maximum level in stationary phase and declines during death phase in various microorganisms. Depletion of nutrient concentration and cellular fragmentation is very common in death phase which results release of intracellular material and proteases in fermentation broth [67, 68]. Increase in toxic waste results a decrease in growth and inactivation of secretary machinery of enzymes [53, 69]. The maximal xylanase production was observed after an incubation time of 2nd day in *Aspergillus fumigatus* F-993 [65] using white corn flour as the substrate under SSF conditions and *Aspergillus flavus* [61] also showed maximal production of xylanase on 2nd day of incubation using 0.5% wheat bran as the substrate under SmF conditions. Pal and Khanum [70] found an incubation time of 6th day for maximal xylanase production (2596 IU/gds) by *Aspergillus niger* using wheat bran as the substrate at 40 °C under SSF conditions. Maximal xylanase production was observed after an incubation period of 7th day for *Coprinellus disseminatus* [71], 8th day for *Volvariella diplasia* [72], and 11th day for *Schizophyllum commune* [58].

2.3.4.2: Effect of incubation temperature on xylanase production

The effect of temperature on maximal xylanase production from both the fungal strains was studied by varying the temperature from 26 to 46°C with a gap of 4°C while keeping other variables constant as shown in Table 2.5. An incubation temperature of 30°C was found optimum to produce maximal xylanase production form *S. commune* ARC-11 (5358.93 IU/gds) and *A. flavus* ARC-12 (1431.19 IU/gds). A deviation from a temperature of 30 °C affected the xylanase production adversely (Figures 2.6 & 2.7). The fungal strain *S. commune* ARC-11 was not able to grow beyond a temperature of 42 °C. Similarly, *A. flavus* ARC-12 showed growth at a temperature of 46 °C and it retained 32.38 and 25.21% of xylanase activity at 42 and 46 °C respectively. Likewise, protein concentration followed the same trend as observed in case of xylanase production for both the fungal strains. At lower temperature, xylanase production decreases due to lower transport of substrates through the cell membrane [70]. The higher temperature leads poor growth due to thermal denaturation of enzymes for metabolic pathways which results the higher maintenance energy for cellular growth and lower metabolites production [71, 73]. de Souza et al. [60] reported the maximum xylanase production by *Aspergillus flavus* at 30 °C using corncob as the substrate under SmF conditions. Shah and

Madamwar [64] also found maximal xylanase production (2701 U/gds) by *Aspergillus foetidus* at 30 °C under SSF conditions after 4th day of incubation. Saleem et al. [74] reported maximal xylanase production at 30 °C by various white-rot fungi such as *Phanerochaete sordida* MRL3 (272.74 IU/ml), *Lentinus pigrinus* MRL6 (278.52 IU/ml) and *Poliporus caliatus* MRL7 (292.86 IU/ml).

2.3.4.3: Effect of initial pH on xylanase production

pH affects the microbial enzyme secretary machinery and therefore, enzyme production. Table 2.6 revealed the effect of initial pH on xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12. The xylanase activity of *S. commune* ARC-11 increased with increasing initial pH from 4.0 to 7.0 and maximal xylanase production (6340.71 IU/gds) was attained at initial pH of 7.0. Beyond a pH of 7.0, xylanase activity started to decrease (Figure 2.8). For *A. flavus* ARC-12, maximum xylanase activity (1663.72 IU/gds) was found at initial pH of 6.0 and on either side of optimum pH (6.0) xylanase activity started to decline (Figure 2.9). The initial pH is found to affect the transport of enzymes across the cell membrane [75]. An increase or decrease in pH, by adjusting to values other than the optimal value, the production of xylanase is found to decrease gradually. In view of the fact that enzymes are proteins, the ionic character of the amino and carboxylic acid groups on the protein surface are liable to be influenced by pH changes and the catalytic properties of the enzymes were strikingly affected. [71]. Most of the xylanase producing fungi grow at a wide pH range varying from 5.0-8.0 [5, 40, 64, 76]. Bhushan et al. [76] reported a pH of 6.0 for maximum xylanase production by *Aspergillus flavus* MTCC9390. Agnihotri et al. [71] observed 6.4 as the optimum pH for xylanase production by a white-rot fungi *Coprinellus disseminatus* under SSF conditions. Muthezhilan et al. [77] found maximum xylanase production (3.72 U/ml) at initial pH of 8.0 by *Penicillium oxalicum* using wheat bran as the carbon source under SSF.

2.3.4.4: Effect of initial moisture content on xylanase production

Table 2.7 revealed the effect of moisture content on xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12. For *S. commune* ARC-11, xylanase production increased with increasing moisture content from 55 to 70%, and reached to its maximum value (6721.96 IU/gds) at 70% moisture contents. Further increase in moisture content (beyond 70%) resulted into poor xylanase production (Figure 2.10). The xylanase activity of 93.16% was

retained at 75% moisture level. For *A. flavus* ARC-12, the maximum xylanase production (1699.50 IU/gds) was observed at a moisture level of 77.5%. For *A. flavus* ARC-12, xylanase activity of 89.90% was retained at 80% moisture content, and beyond that a sharp decline in xylanase activity was observed (Figure 2.11). Microbial growth and product synthesis depends on physical characteristics of substrate which is affected by moisture content during SSF. Moisture content causes the swelling of substrate and makes the substrate suitable for microbial utilization and growth. The appropriate moisture content results the faster microbial growth and early initiation of enzyme production [53, 64, 78, 79]. The moisture content higher than optimum level causes the decreased porosity, alteration in particle structure, and gummy texture which limits the oxygen transfer into the substrate [53, 64, 80]. Moreover, the risk of microbial contamination increases due to sticking of substrate particles to the wall of the reactor or conglomeration of substrate [64, 81]. If the moisture content is lower than optimum level then water tension becomes high and solubility of nutrients in substrate as well as degree of swelling of substrate starts to decline [53, 82]. Ghanem et al. [83] studied the effect of moisture content (25 to 85%) on xylanase production by *Aspergillus terreus* and reported optimum xylanase activity at 75% of moisture content. Similar observations were made by Pal and Khanum [70] who tested the effect moisture content on xylanase production varied from 55 to 80% and found maximum xylanase production (more than 1650 IU/gds) by *Aspergillus niger* at 70% moisture content under SSF conditions. Maciel et al. reported maximum xylanase production (3099 IU/g) at a moisture level of 84% by *Aspergillus niger* LPB326 under SSF conditions [84].

2.3.4.5: Effect of nitrogen sources on xylanase production

Among simple nitrogen sources, ammonium sulphate (0.08% as available N) followed by urea (0.08% as available N) were found most effective nitrogen sources for xylanase production by *S. commune* ARC-11 (Figure 2.12). Among simple nitrogen sources, ammonium sulphate produced maximum xylanase activity of 8591.38 IU/gds and followed by urea (8123.20 IU/gds) using rice straw as the source of carbon under SSF conditions (Table 2.8). Among complex nitrogen sources, maximum xylanase production was found with beef extract (8221.60 IU/gds) and followed by peptone (7787.82 IU/gds) at a concentration of 0.8% (w/v) (Figure 2.13). Likewise, ammonium sulphate (0.08% as available N) was found to produce maximum xylanase activity (2014.85 IU/gds) in case of *A. flavus* ARC-12 using pearl millet

stover as the source of carbon under SSF conditions (Table 2.9 & Figure 2.14). For *A. flavus* ARC-12, beef extract (1.2%, w/v) was observed to produce maximum xylanase activity (2219.85 IU/gds) compared to other complex nitrogen sources as well as all the simple nitrogen sources (Figure 2.15). Production of xylanases is known to be sensitive to nature of nitrogen source used and the percentage of available nitrogen used in production medium [85]. Ammonium sulphate was used as the best and cost effective nitrogen source for xylanase production by *S. commune* ARC-11 [79]. Ammonium sulphate was also reported to produce maximum xylanase production by some other fungi such as *Aspergillus terreus* [83], and *Thermoascus aurantiacus* [79]. Laxmi et al. [86] found 1% beef extract as the most suitable nitrogen source for maximum xylanase production (6248 IU/ml) by *Aspergillus* sp. RSP6. Kumar [87] also observed maximum xylanase production (687.97 IU/mL) by *Coprinus cinereus* using beef extract (1.0 g/l) as the source of complex nitrogen under SSF conditions on wheat bran medium.

2.3.4.6: Effect of surfactants on xylanase production

Among various surfactants tested, *S. commune* ARC-11 produced maximum xylanase production ((10196.53 IU/gds)) with Tween-20 (0.10%, w/v) which was followed by other surfactants in descending order i.e. Tween-80 (0.10%, w/v), Tween-60 (0.10%, w/v), Tween-40 (0.10%, w/v) and Triton-x-100 (0.05%, w/v) for (Table 2.10 & Figure 2.16). Similarly, for *A. flavus* ARC-12, Tween-60 (0.10%, w/v) produced maximum xylanase activity (2539.54 IU/gds) which was followed in descending order by Tween-80 (0.10%, w/v), Tween-40 (0.10%, w/v) and Triton-x-100 (0.05%, w/v) (Figure 2.17). EDTA and SDS showed inhibitory effect on xylanase production for both the fungal strains *S. commune* ARC-11 and *A. flavus* ARC-12. The stimulatory effect of surfactants on enzyme production is well established [88-91]. Surfactants promote the water penetration into the solid substrate matrix which improves available surface area due to swelling for microbial growth [88, 89, 92]. Surfactants are also found to enhance the permeability of microbial cell membrane which affects the secretion of certain proteins. Surfactants also assist the release of cell-bound enzymes into the fermentation broth [90, 93]. Similar results were observed by Pandya et al. [90] who reported improvement in xylanase production by *Aspergillus tubingensis* JP-1 using different surfactants such as Triton-x-100, Tween-80, Tween-60, Tween-40, and Tween-20. Improvement in xylanase production by various microorganisms was also reported with different surfactants such as

Tween-20 for *Cellulosimicrobium cellulans* CKMX1 [94], Tween-80 for *Trichoderma viride*-IR05 [91], *Aspergillus niger* NRC 107 [95], *Fusarium oxysporum* [96], and Triton-x-100 for *Aspergillus tubingensis* JP-1 [90].

S. commune ARC-11 produced maximum xylanase (1147.11 IU/ml), cellulase (1.47 IU/ml) and laccase (28.65 U/ml) activities at optimum cultural conditions such as incubation time 8th day, temperature 30 °C, pH 7.0, moisture content 70.0%, nitrogen source (ammonium sulphate, 0.08% as available nitrogen), and surfactant (Tween-20, 0.10% (w/v)) using rice straw as the carbon source under SSF conditions. *A. flavus* ARC-12 produced maximum xylanase production (234.26 IU/ml) at optimum cultural conditions such as incubation time 48 h, temperature 30 °C, pH 6.0, moisture content 77.5%, nitrogen source (beef extract, 1.2% (w/v)), and surfactant (Tween-60, 0.10% (w/v)) using pearl millet stover as the carbon source under SSF conditions (Table 2.11). Cellulase and laccase activities were not detected in the crude enzyme from *A. flavus* ARC-12.

2.3.5. Bio-chemical characterization of xylanases

2.3.5.1. Partial purification of xylanases

Xylanases produced at optimum cultural conditions by *S. commune* ARC-11 and *A. flavus* ARC-12 were subjected to ammonium sulphate precipitation for partial purification. The maximum xylanase activity was recorded at 50-70% ammonium sulphate fraction by both the fungal strains *S. commune* ARC-11 and *A. flavus* ARC-12. For *S. commune* ARC-11, a yield of 41.86% with 2.75 fold purification was obtained (Table 2.12). For *A. flavus* ARC-12, a yield of 45.05% with fold purification of 2.85 was achieved. Ammonium sulphate precipitation which is a low cost technique for partial purification of proteins has been used widely for partial purification of xylanases [83, 97, 98]. Bhushan et al. [98] performed ammonium sulphate precipitation for xylanase purification from *Aspergillus flavus* MTCC 9390 and attained the yield of 23.6% with fold purification of 3.89 by 30-70% of ammonium sulphate. Ghanem et al. [83] studied xylanase purification from *Aspergillus terreus* and purified xylanase 1.5 times with the yield of 34.5% by ammonium sulphate precipitation.

2.3.5.2. Effect of pH on xylanase activities

For *S. commune* ARC-11, the maximum xylanase activity was found at pH 5.0 for and results clearly showed the xylanase was active at a broad pH range of 4.0-7.0. The xylanase

activities of 84.17 and 55.24% were retained at pH 6.0 and 7.0 respectively while at pH 8.0 only 26.66% of xylanase activity was retained (Table 2.13). Xylanase from *A. flavus* ARC-12 was also active at a broad range of pH (3.0-9.0) and 30.02% of xylanase activity was retained at pH 3.0. The xylanase from *A. flavus* ARC-12 was found stable in alkaline pH range, showing the alkali-tolerant nature of enzyme. 92.30, 52.69 and 40.64% of xylanase activities were retained at pH 7.0, 8.0 and 9.0 respectively (Figure 2.18). The stability of xylanase in alkaline pH range made it suitable for bio-bleaching of pulp in alkaline conditions. Kolenova et al. [58] studied the effect of pH on xylanase from *Schizophyllum commune* and reported similar observation. The xylanase from *Schizophyllum commune* was stable at a pH range of 4.0-7.0 with optimum activity at pH 5.5. Bhushan et al. [98] reported the optimum pH of 5.0 for xylanase obtained from *Aspergillus flavus* and 75% of its activity was retained at pH 7.0 and after that xylanase activity declined sharply. Chidi et al. [99] also reported the xylanase obtained from *Aspergillus terreus* UL 4209 which was most active at pH 6.0 and activity decreased significantly towards alkaline pH.

2.3.5.3. Effect of temperature on xylanase activities

Xylanases from *S. commune* ARC-11 and *A. flavus* ARC-12 exhibited activity over a broad temperature range of 30-65 °C (Table 2.14). Xylanase from *S. commune* ARC-11 showed maximal activity at 55 °C and retained 47.06, 47.25, and 24.78% of maximum xylanase activity at 30, 65, and 70 °C respectively. Xylanases from *A. flavus* ARC-12 was found thermo-tolerant with optimum xylanase activity at 50 °C. Xylanases activity from *A. flavus* ARC-12 increased with increasing temperature up to 50 °C, but beyond that declined progressively and retained 43.62% of xylanase activity at 70 °C (Figure 2.19). The maximum activity of xylanase at 50 °C was reported for several fungi such as *Schizophyllum commune* [58], *Aspergillus terreus* [83], *Aspergillus ochraceus* [100], and *Fusarium verticillioides* [101]. The maximum activity of xylanase at 55 °C was reported for fungal strains like *Aspergillus versicolor* [102], *Rhizopus oryzae* [103], and *Acrophialophora nainiana* [104].

2.3.5.4. Thermo-stability of xylanase

Thermo-stability xylanases from *S. commune* ARC-11 and *A. flavus* ARC-12 were studied at different temperatures, holding times and optimum pH (Table 2.15). Xylanase from *S. commune* ARC-11 was stable at temperature 45 and 50 °C for 180 min while at 55 °C (optimum temperature) xylanase activity decreased slightly after 120 min. At temperature 60

°C xylanase activity decreased drastically and only 24.29% of activity was retained after 180 min of holding time. For *A. flavus* ARC-12, xylanase was stable at temperature 45 and 50 °C up to 180 min while at 55 and 60 °C temperature xylanase activity declined drastically after 60 min of holding time. Xylanases from *S. commune* ARC-11 and *A. flavus* ARC-12 stability for a longer time at their respective optimum temperatures was important for their application in prebleaching studies. Guimaraes et al. [61] observed a half life of more than 75 and 45 min for xylanase from *Aspergillus flavus* at 50 and 55 °C temperature respectively. Chidi et al. [99] studied the thermo-stability of xylanase from *Aspergillus terreus* UL 4209 and found half life of 5.8 h at 50 °C.

2.3.5.5. Effect of metal ions on partially purified xylanase

There are different sources of metal ions entering in the pulp and paper manufacturing process i.e. water, lime and white liquor which may affect the enzyme activity during pre-bleaching of pulp [105]. With this consideration, the effect of metal ions on xylanase activities was also studied at two different concentration levels i.e. 1 and 10 mM. Metal ions including Na^{+} and K^{+} showed stimulatory effect on xylanase activity whereas other metal ions had inhibitory effect on xylanase activity from *S. commune* ARC-11 (Figure 2.20). For *A. flavus* ARC-12, metal ions such as Na^{+}, K^{+}, Al^{+++}, Ca^{++}, Co^{++}, Mg^{++}, and Zn^{++} showed stimulatory effect on xylanase activity (Table 2.16). Xylanase activity was increased by 66.03, 62.19, and 39.69% by the addition of Na^{+}, K^{+} and Zn^{++} respectively at 10 mM concentration. Cu^{++}, Fe^{++}, Mn^{++}, and Pb^{++} were found to inhibit the xylanase activity for *A. flavus* ARC-12 (Figure 2.21). Hg^{++} (1 and 10 mM) strongly inhibited the xylanase activity for both the fungal strains *S. commune* ARC-11 and *A. flavus* ARC-12. These results go with the findings of Ghanem et al. [83] who reported inhibition in xylanase activity obtained from *Aspergillus terreus* by the addition of metal ions like Hg^{++}, Co^{++}, Cu^{++}, Fe^{+++}, and Pb^{++}. In earlier studies, Hg^{++} was reported as the strong inhibitor of xylanase activity and metal ions such as Cd^{++}, Cu^{++}, Fe^{++}, Mn^{++}, and Pb^{++} were also commonly cited as inhibitors of xylanases production [83, 98, 106]. Enzyme activity is probably inhibited through attack on certain groups at the active sites of enzymes. Inactivation of xylanases by Cu^{++} and Hg^{++} probably indicated the presence of thiol and histidine as the active site residues [100, 107]. Stimulatory effect of Ca^{++} on xylanase activity from *A. flavus* ARC-12 indicated its possible role as co-factor in enzyme–substrate reaction and showed the stabilizing effect [83, 108].

Table-2.1: Screening of xylanase producing fungi

Fungal isolate	Source	Site of isolation	Xylanase activity (IU/ml)	Cellulase activity (IU/ml)
ARC-2	Dead and decaying wood	Saharanpur, Uttar Pradesh	95.33±4.92	1.26±0.05
ARC-3	Dead and decaying wood	Saharanpur, Uttar Pradesh	118.44±4.85	2.28±0.10
ARC-5	Lignocellulose rich soil	Paonta Sahib, Himachal Pradesh	152.88±5.27	2.65±0.12
ARC-6	Lignocellulose rich soil	Paonta Sahib, Himachal Pradesh	41.71±2.14	0.63±0.03
ARC-9	Lignocellulose rich soil	Paonta Sahib, Himachal Pradesh	63.55±2.28	1.30±0.07
ARC-10	Dead and decaying wood	Paonta Sahib, Himachal Pradesh	118.20±3.55	2.60±0.13
ARC-11	**Dead and decaying wood**	**Paonta Sahib, Himachal Pradesh**	**386.44±8.76**	**1.05±0.05**
ARC-12	**Soil rich in lignocellulose**	**Jaipur, Rajasthan**	**102.66±4.19**	**ND***
ARC-16	Lignocellulose rich soil	Jaipur, Rajasthan	127.42±5.11	2.54±0.11
ARC-20	Dead and decaying wood	Jaipur, Rajasthan	114.20±4.22	2.66±0.13
ARC-22	Dead and decaying wood	Jaipur, Rajasthan	57.86±1.34	1.71±0.06
ARC-24	Soil rich in lignocellulose	Dehradun, Uttarakhand	47.04±2.37	1.85±0.07
ARC-25	Soil rich in lignocellulose	Dehradun, Uttarakhand	146.66±4.86	3.35±0.16
ARC-30	Soil rich in lignocellulose	Dehradun, Uttarakhand	218.42±8.40	6.43±0.22
ARC-33	Soil rich in lignocellulose	Dehradun, Uttarakhand	121.57±5.28	2.14±.08
ARC-37	Soil rich in lignocellulose	Dehradun, Uttarakhand	95.60±3.92	1.82±0.07
ARC-38	Soil rich in lignocellulose	Muzaffarnagar, Uttar Pradesh	48.55±2.88	0.81±0.03
ARC-39	Dead and decaying wood	Muzaffarnagar, Uttar Pradesh	86.33±3.77	1.74±0.09
ARC-42	Dead and decaying wood	Muzaffarnagar, Uttar Pradesh	74.22±3.60	1.05±0.04
ARC-44	Dead and decaying wood	Muzaffarnagar, Uttar Pradesh	115.10±3.52	2.24±0.10

*Not detected

± refers standard deviation

Table-2.2: Effect of carbon sources for xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12

S. No.	Carbon sources	Xylanase activity (IU/gds)	
		***S. commune* ARC-11**	***A. flavus* ARC-12**
1	Corn cob	2812.80±88.32	704.32±16.20
2	Corn stover	2848.36±69.50	632.12±13.08
3	Congress grass	3235.56±80.89	1187.17±54.49
4	Maize bran	3962.40±141.46	1216.00±48.40
5	**Pearl millet stover**	**2955.02±109.34**	**1345.44±57.72**
6	Sabai grass	3681.96±138.07	888.40±25.76
7	Sugarcane bagasse	3642.40±150.07	836.36±22.83
8	Sugarcane leaves	3460.71±79.60	1238.66±46.57
9	**Rice straw**	**4288.36±143.66**	**1215.03±42.16**
10	Wheat bran	3587.11±118.02	1161.15±37.16
11	Wheat straw	3771.82±112.40	767.96±16.20

± refers standard deviation

Table-2.3: Effect of incubation time on xylanase production by *S. commune* ARC-11

Incubation time (days)	Xylanase activity (IU/gds)	Protein content (mg/ml)
2	256.00±7.99	0.85±0.03
3	338.93±10.85	0.97±0.04
4	1389.07±35.42	1.11±0.03
5	1490.93±40.85	1.44±0.05
6	4262.76±153.46	1.69±0.06
7	4556.98±212.36	1.73±0.06
8	**5199.02±132.06**	**1.86±0.08**
9	4039.91±148.67	1.99±0.06
10	2898.93±93.93	1.96±0.08
11	1747.73±44.39	1.95±0.07
12	1472.80±33.87	1.90±0.06

± refers standard deviation

Table-2.4: Effect of incubation time on xylanase production by *A. flavus* ARC-12

Incubation time	Xylanase activity (IU/gds)	Protein content (mg/ml)
12	298.23±10.56	0.57±0.03
24	597.00±25.79	1.03±0.05
36	1190.53±34.88	1.15±0.06
48	1424.69±64.82	1.18±0.06
60	1385.66±65.40	1.22±0.05
72	1326.14±68.96	1.19±0.04
84	1136.32±49.88	1.17±0.05
96	1039.73±38.47	1.15±0.04

± refers standard deviation

Table-2.5: Effect of temperature on xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12

Temperature (°C)	*S. commune* ARC-11		*A. flavus* ARC-12	
	Xylanase activity (IU/gds)	Protein content (mg/ml)	Xylanase activity (IU/gds)	Protein content (mg/ml)
26	4001.96±158.08	1.53±0.09	1305.22±56.52	1.02±0.06
30	5358.93±253.48	1.88±0.09	1431.19±54.53	1.20±0.05
34	3689.87±143.17	1.58±0.07	1020.98±42.88	1.14±0.06
38	1829.07±88.89	1.50±0.08	609.46±24.01	1.06±0.07
42	—	—	463.55±18.45	0.93±0.05
46	—	—	360.89±17.32	0.72±0.04

± refers standard deviation

Table-2.6: Effect of initial pH on xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12

Initial pH	*S. commune* ARC-11		*A. flavus* ARC-12	
	Xylanase activity (IU/gds)	Protein content (mg/ml)	Xylanase activity (IU/gds)	Protein content (mg/ml)
4.0	5242.40±144.69	1.65±0.09	1291.24±48.68	1.12±0.05
5.0	5299.73±171.18	1.92±0.09	1491.68±67.13	1.18±0.06
5.5	5459.73±191.09	1.98±0.12	1589.03±81.04	1.33±0.09
6.0	5661.16±157.38	2.17±0.11	1663.72±77.03	1.48±0.08
6.5	5973.33±221.01	2.24±0.10	1547.62±59.74	1.55±0.08
7.0	6340.71±207.34	2.25±0.12	1456.99±57.41	1.46±0.09
8.0	5424.18±227.82	2.12±0.11	1408.86±64.10	1.40±0.08
9.0	4934.31±188.49	1.94±0.10	1369.83±64.79	1.22±0.06
10.0	4811.82±188.62	1.90±0.12	1259.91±61.74	1.20±0.06

± refers standard deviation

Table-2.7: Effect of initial moisture content on xylanase production by *S. commune* ARC11 and *A. flavus* ARC12

Initial moisture content (%)	*S. commune* ARC-11		*A. flavus* ARC-12	
	Xylanase activity (IU/gds)	Protein content (mg/ml)	Xylanase activity (IU/gds)	Protein content (mg/ml)
55.0	3026.13±86.24	1.32±0.07	960.70±40.54	1.05±0.05
60.0	4118.49±130.14	1.56±0.08	1021.41±38.30	1.11±0.05
65.0	5459.73±177.44	1.83±0.10	1097.95±29.97	1.36±0.07
70.0	6721.96±235.27	2.14±0.10	1321.59±54.85	1.44±0.07
75.0	6262.67±180.36	2.30±0.12	1599.55±70.06	1.48±0.07
77.5	4428.62±217.00	2.21±0.12	1699.50±56.59	1.51±0.08
80.0	2054.31±87.51	2.15±0.11	1528.00±55.62	1.40±0.07
82.5	1619.73±51.83	1.92±0.10	1283.76±53.92	1.26±0.06
85.0	1303.64±49.80	1.87±0.09	1041.36±33.22	1.22±0.06

± refers standard deviation

Table-2.8: Effect of nitrogen sources on xylanase production by *S. commune* ARC-11

	Particulars	Nitrogen sources (as percent available nitrogen)			
Simple nitrogen sources		0.04 % N	0.08 % N	0.12 % N	0.16 % N
NH_4Cl	Xylanase	6814.76±175.82	7141.87±267.11	6513.78±276.84	6437.87±227.26
	Protein content	3.30±0.16	3.43±0.15	4.18±0.19	4.32±0.16
NH_4NO_3	Xylanase	5304.89±222.27	5859.56±246.10	5262.22±178.92	4930.31±192.28
	Protein content	3.25±0.11	3.49±0.10	3.55±0.14	3.64±0.15
$(NH4)_2PO_4$	Xylanase	6013.60±247.16	6994.96±225.94	7552.00±311.14	6872.89±267.36
	Protein content	3.0±0.10	3.69±0.15	3.89±0.17	4.15±0.19
$(NH4)_2SO_4$	Xylanase	7414.49±235.78	8591.38±358.26	7781.87±309.72	7354.04±325.78
	Protein content	3.35±0.14	3.65±0.12	3.67±0.14	4.26±0.20
$NaNO_3$	Xylanase	6193.78±261.38	6926.22±248.65	6684.44±282.75	5641.42±233.55
	Protein content	2.63±0.11	2.89±0.10	2.80±0.14	2.66±0.14
Urea	Xylanase	7594.04±349.33	8123.20±311.12	7982.76±344.86	7720.27±339.69
	Protein content	3.17±0.14	3.16±0.12	3.70±0.16	3.45±0.17
Complex nitrogen sources		Nitrogen sources, (w/v)			
		0.4 %	0.8 %	1.2 %	1.6 %
Beef	Xylanase	7396.62±181.96	8221.60±305.84	7688.27±323.68	5707.20±215.16
	Protein content	3.32±0.15	4.40±0.14	4.06±0.17	4.10±0.17
Malt	Xylanase	5377.16±266.71	5840.53±412.93	5718.49±196.72	5028.71±288.15
	Protein content	3.09±0.09	3.13±0.14	3.19±0.13	3.56±0.17
Peptone	Xylanase	7222.49±252.06	7787.82±379.27	7741.60±305.02	7559.11±271.37
	Protein content	3.03±0.14	3.49±0.11	3.49±0.15	4.14±0.20
Soyabean	Xylanase	5056.00±173.93	6180.71±259.59	5868.98±255.30	4871.11±203.13
	Protein content	3.04±0.14	3.17±0.12	3.56±0.17	2.89±0.14
Tryptone	Xylanase	4901.87±225.98	5935.38±235.04	6030.22±233.37	5979.82±263.11
	Protein content	2.66±0.14	3.53±0.13	4.17±0.19	4.70±0.21
Yeast	Xylanase	6602.67±283.91	7412.09±329.10	7985.78±391.30	7687.11±282.12
	Protein content	3.48±0.11	4.45±0.19	4.70±0.19	4.83±0.21
Control	Xylanase	4954.04±216.99			
	Protein content	2.06±0.11			

± refers standard deviation

Table-2.9: Effect of nitrogen sources on xylanase production by *A. flavus* ARC-12

	Particulars	Nitrogen sources (as percent available nitrogen)			
Simple nitrogen sources		0.04 % N	0.08 % N	0.12 % N	0.16 % N
NH_4Cl	Xylanase	1562.47±54.37	1772.89±82.62	1743.84±83.36	1475.64±54.75
	Protein content	2.45±0.11	2.95±0.12	3.27±0.14	2.69±0.14
NH_4NO_3	Xylanase	1537.86±65.05	1829.91±97.72	1683.13±83.31	1662.21±77.46
	Protein content	2.42±0.14	2.93±0.09	3.05±0.15	2.84±0.14
$(NH4)_2PO_4$	Xylanase	1457.42±69.37	1688.55±74.97	1609.95±56.51	1599.22±72.28
	Protein content	2.73±0.12	2.77±0.16	3.45±0.15	3.37±0.14
$(NH4)_2SO_4$	Xylanase	1806.71±40.11	2014.85±106.18	1866.12±83.04	1734.84±57.77
	Protein content	2.26±0.13	2.47±0.12	2.91±0.16	2.93±0.17
$NaNO_3$	Xylanase	1378.94±54.33	1515.53±63.34	1411.68±71.14	1156.27±56.07
	Protein content	2.33±0.10	2.84±0.11	3.02±0.16	2.45±0.13
Urea	Xylanase	1708.28±79.09	1978.86±87.27	1722.15±63.38	1695.92±59.70
	Protein content	2.63±0.12	3.41±0.15	3.68±0.13	3.22±0.14
Complex nitrogen sources		Nitrogen sources, (w/v)			
		0.4 %	0.8 %	1.2 %	1.6 %
Beef	Xylanase	1876.63±64.37	2042.82±96.01	2219.85±116.32	1912.73±83.97
	Protein content	3.51±0.20	3.77±0.17	3.83±0.15	3.23±0.17
Malt	Xylanase	1392.38±65.72	1511.63±47.77	1347.83±36.93	1131.77±43.35
	Protein content	3.41±0.16	3.48±0.13	3.55±0.17	3.53±0.16
Peptone	Xylanase	1841.62±84.16	2021.14±70.74	1872.95±45.51	1709.15±84.77
	Protein content	3.21±0.16	3.29±0.14	3.85±0.19	3.51±0.17
Soybean	Xylanase	1221.31±28.58	1563.45±64.26	1302.08±54.17	1307.39±49.42
	Protein content	2.48±0.11	3.70±0.22	4.02±0.17	4.05±0.23
Tryptone	Xylanase	1663.40±74.02	1675.43±59.14	1921.19±94.91	1801.94±85.59
	Protein content	3.21±0.18	3.24±0.15	3.13±0.18	2.91±0.13
Yeast	Xylanase	1650.17±71.45	1865.79±90.86	1785.03±57.48	1685.08±54.09
	Protein content	2.06±0.11	2.33±0.10	3.24±0.11	3.45±0.15
Control	Xylanase	1312.92±46.61			
	Protein content	1.58±0.07			

± refers standard deviation

Table-2.10: Effect of surfactants on xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12

Surfactants	Surfactants at different doses (%, w/v)			
	0.05	**0.10**	**0.20**	**0.30**
***S. commune* ARC-11**				
T-20	9163.38±286.81	10196.53±444.57	10131.29±33.28	9430.04±308.36
T-40	8714.04±318.06	8755.556±302.07	8015.733±19.38	7670.04±243.91
T-60	8697.24±367.02	9033.067±351.39	8356.533±28.45	8072.17±305.13
T-80	8971.82±281.72	9272.089±358.83	8999.467±27.88	8568.88±349.61
T-x-100	8643.91±376.01	8481.956±371.51	8173.778±31.69	8039.46±253.24
SDS	5795.56±199.37	5718.489±205.87	5673.067±22.65	5566.40±181.46
EDTA	6032.53±183.99	5825.156±186.40	5056.711±19.17	4389.06±170.30
Control	6680.44±225.80			
***A. flavus* ARC-12**				
T-20	1918.81±54.88	2030.79±92.81	1789.37±61.91	1838.58±77.04
T-40	2012.04±88.53	2327.07±88.66	2275.79±89.89	1977.35±72.96
T-60	2363.92±89.36	2539.54±78.22	2476.02±77.75	2245.44±73.87
T-80	2102.34±101.96	2372.60±88.26	2279.37±98.01	2071.98±77.70
T-x-100	2022.12±77.65	1905.04±64.58	1537.86±58.44	1480.08±54.47
SDS	1626.11±59.52	1600.09±58.72	1498.84±50.96	927.96±35.36
EDTA	1767.69±84.14	1665.13±62.78	1641.93±53.69	1477.15±58.35
Control	1910.35±62.47			

± refers standard deviation

Table-2.11: Optimized conditions for xylanase production *S. commune* ARC-11 and *A. flavus* ARC-12

Particulars	***S. commune* ARC-11**	***A. flavus* ARC-12**
Cultural parameters		
Carbon source	Rice straw	Pearl millet stover
Incubation time (days)	8	2
Temperature (°C)	30	30
pH	7.0	6.0
Moisture content (%)	70.0	77.5
Nitrogen source (%)	Ammonium sulphate (0.08 % N)	Beef extract (1.2 % W/V)
Surfactant (%)	Tween-20 (0.10 % w/v)	Tween-60 (0.10 % w/v)

Table-2.12: Partial purification of xylanases from *S. commune* ARC-11 and *A. flavus* ARC-12

Fungal strains	Steps	Volume (ml)	Activity (IU/ml)	Total activity (IU)	Protein (mg/ml)	Total protein (mg)	Specific activity (IU/mg)	Yield (%)	Fold purification
***S.commune* ARC-11**	Crude enzyme	200.0	1169.60	233920.0	5.09	1018.00	229.78	100	1
	$(NH_4)_2SO_4$ (50-70%)	17.0	5760.50	97928.50	9.11	154.90	632.18	41.86	2.75
***A.flavus* ARC-12**	Crude enzyme	200.0	224.82	44964.00	2.62	524.00	85.80	100	1
	$(NH_4)_2SO_4$ (50-70%)	8.5	2383.52	20259.92	9.74	82.83	244.59	45.05	2.85

Table-2.13: Effect of pH on xylanase activity from *S. commune* ARC-11 and *A. flavus* ARC-12

pH	Relative xylanase activity (%)	
	***S. commune* ARC-11**	***A. flavus* ARC-12**
3.0	29.58±2.16	30.02±2.56
4.0	58.63±3.80	61.09±3.05
5.0	100.00±3.26	92.64±3.28
5.5	88.14±3.48	95.56±3.64
6.0	84.17±2.65	100.00±3.25
6.5	64.60±2.77	93.04±2.70
7.0	55.24±3.52	92.30±2.61
8.0	26.66±2.18	52.69±2.12
9.0	24.19±2.09	40.64±2.10

± refers standard deviation

Table-2.14: Effect of temperature on xylanase from *S. commune* ARC-11 and *A. flavus* ARC-12

Temperature (ºC)	Relative xylanase activity (%)	
	***S. commune* ARC-11**	***A. flavus* ARC-12**
30	47.06±2.82	45.90±2.73
35	55.41±3.10	51.60±2.26
40	67.84±2.96	56.59±3.18
45	82.96±3.38	91.13±2.85
50	97.05±3.45	100.00±2.64
55	100.00±3.19	93.58±3.14
60	71.27±2.94	74.75±2.82
65	47.25±2.90	59.29±3.55
70	24.78±2.43	43.62±2.44

± refers standard deviation

Table-2.15: Thermo-stability of xylanases from *S. commune* ARC-11 and *A. flavus* ARC-12 at optimum pH

Holding time (min)	Relative xylanase activities at different temperature (°C)			
	45	**50**	**55**	**60**
***S. commune* ARC-11**				
0	100.00	100.00	100.00	100.00
30	100.00	98.72	98.18	85.63
60	99.47	98.36	97.45	71.94
90	98.24	96.84	93.73	53.16
120	96.73	94.35	89.26	38.75
180	95.28	92.19	84.38	24.29
***A. flavus* ARC-12**				
0	100.00	100.00	100.00	100.00
30	99.34	98.78	86.43	77.14
60	97.72	95.27	72.27	58.33
90	96.11	90.25	60.56	36.78
120	94.35	87.66	44.43	24.39
180	92.56	84.75	38.83	15.54

Table-2.16: Effect of metal ions on xylanase activity from *S. commune* ARC-11 and *A. flavus* ARC-12

Metal ions	Relative xylanase activity (%)			
	***S. commune* ARC-11**		***A. flavus* ARC-12**	
	1 mM	10 mM	1 mM	10 mM
Na^{+}	124.91±3.75	124.29±3.52	168.69±3.10	166.03±2.22
K^{+}	115.95±2.90	123.36±3.57	163.66±3.18	162.19±2.10
Al^{+++}	90.98±3.66	62.30±2.88	158.94±2.33	148.84±2.36
Ca^{++}	102.22±3.17	100.56±3.16	154.80±2.45	139.59±3.05
Cd^{++}	59.89±2.98	44.38±2.55	94.09±3.68	46.51±2.68
Co^{++}	95.24±3.50	67.06±2.93	136.93±3.22	133.68±3.47
Cu^{++}	95.86±2.85	63.78±2.84	95.42±3.25	52.73±2.39
Fe^{++}	46.85±2.09	33.31±2.02	94.67±3.15	40.47±3.27
Hg^{++}	0	0	9.16±1.85	0
Mn^{++}	47.34±2.44	43.39±2.19	66.54±2.62	32.50±2.18
Mg^{++}	97.65±3.53	52.90±2.58	115.21±3.40	111.23±3.51
Ni^{++}	96.35±3.77	55.50±2.86	94.09±2.75	88.48±3.35
Pb^{++}	57.91±2.95	55.75±3.05	82.57±3.89	79.62±3.20
Zn^{++}	74.17±3.11	58.84±3.12	161.74±3.25	139.69±3.24
Control	100±2.42	100±3.44	100±3.14	100±3.26

± refers standard deviation

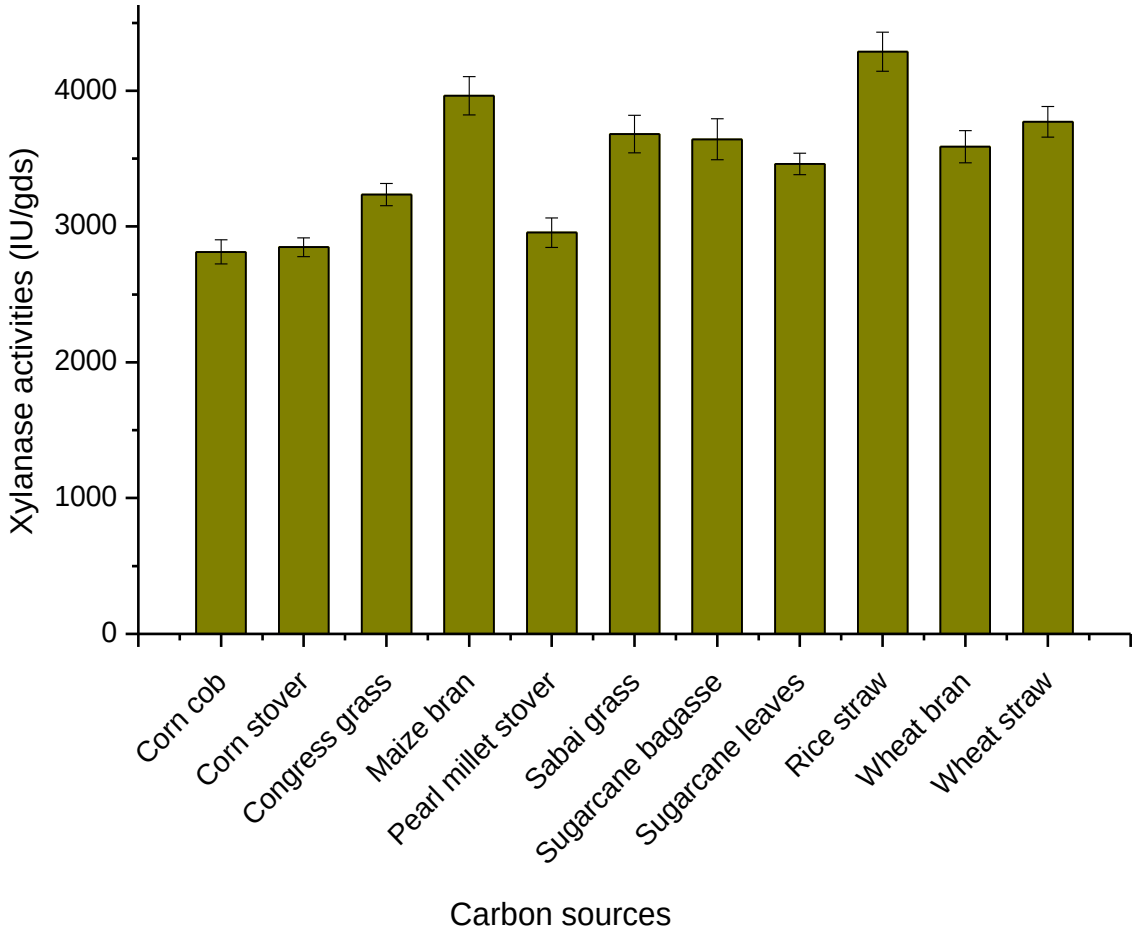

Figure-2.2: Effect of carbon sources on xylanase production by *S. commune* ARC-11

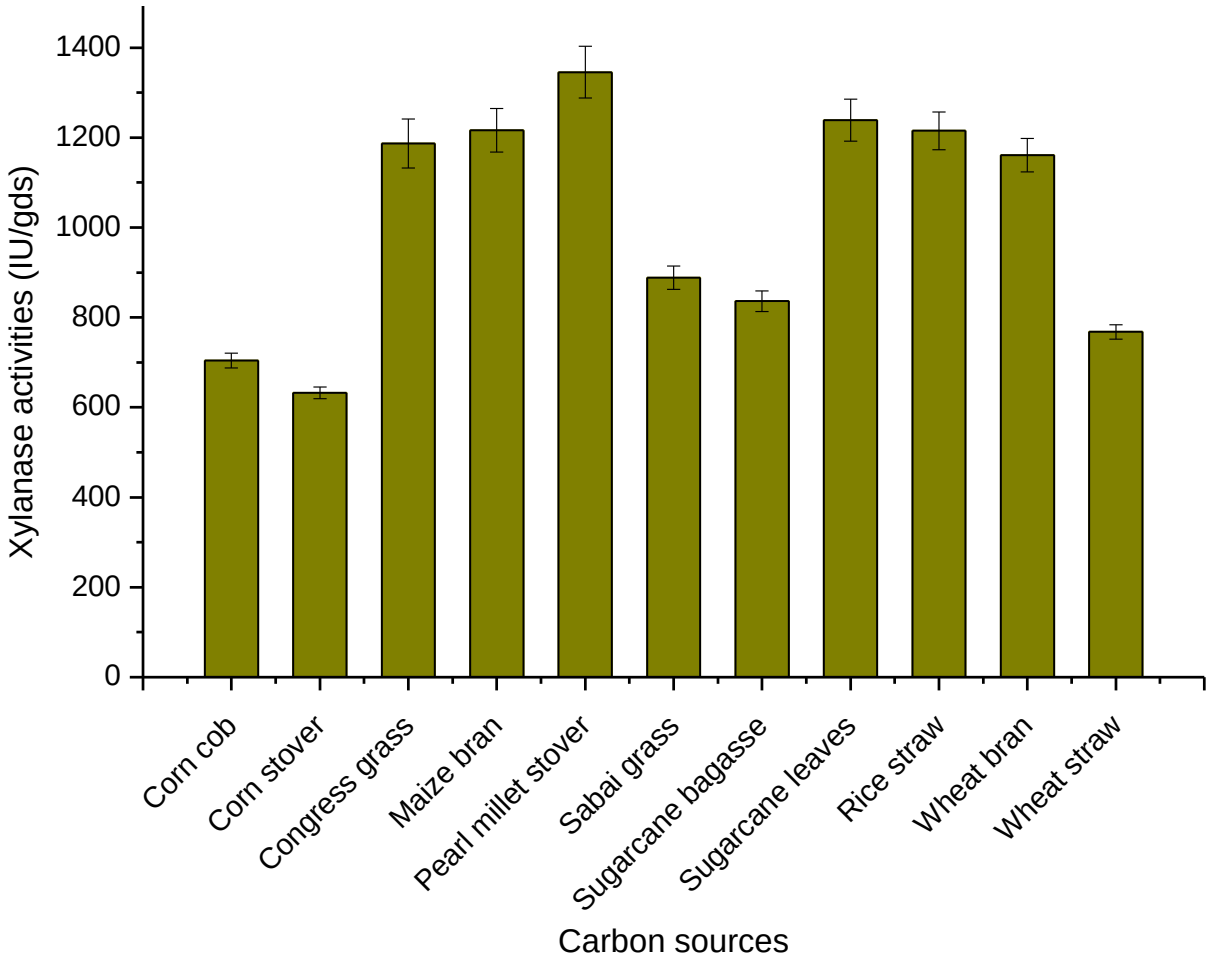

Figure-2.3: Effect of carbon sources on xylanase production by *A. flavus* ARC-12

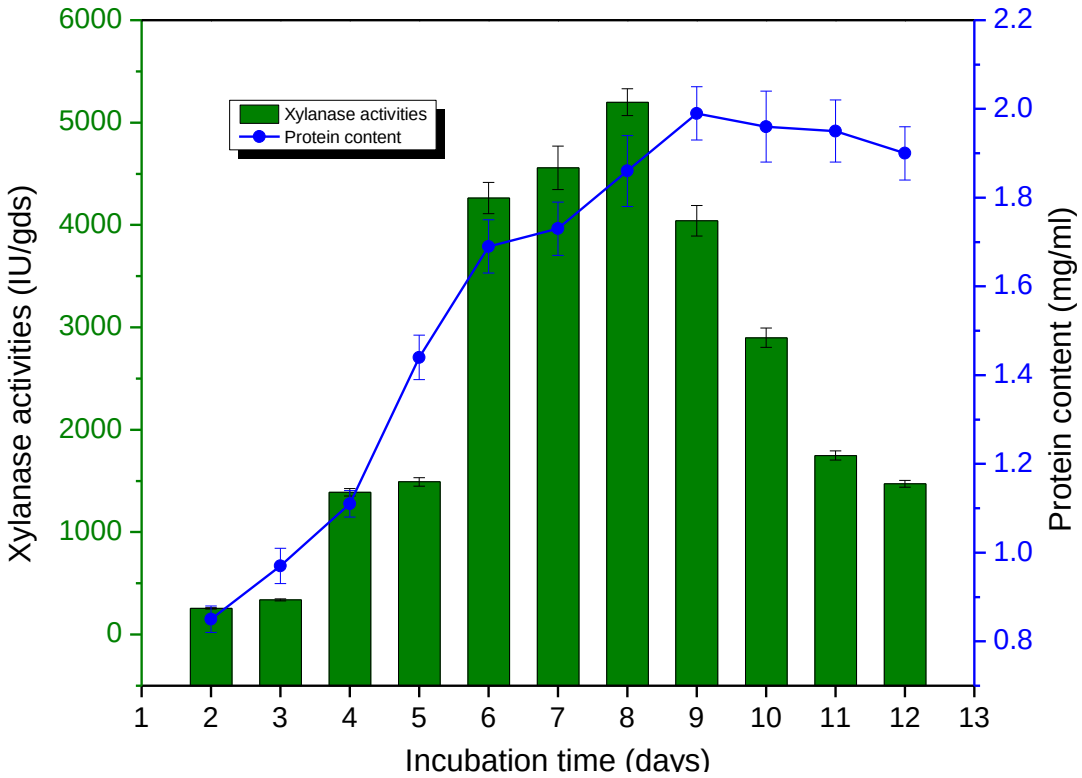

Figure 2.4 Effect of incubation time on xylanase production by *S. commune*-ARC-11

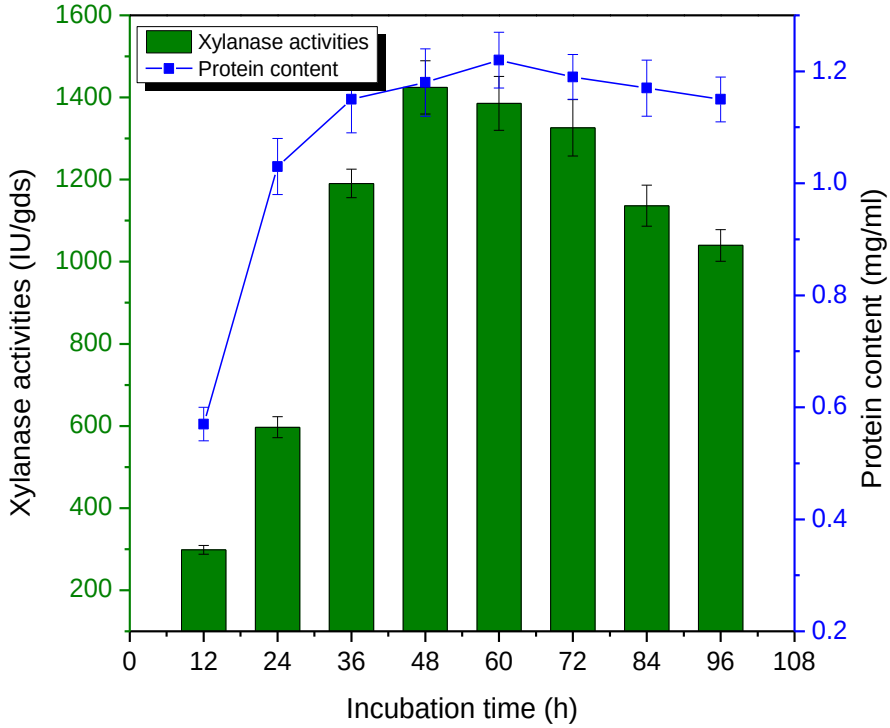

Figure-2.5: Effect of incubation time on xylanase production by *A. flavus* ARC-12

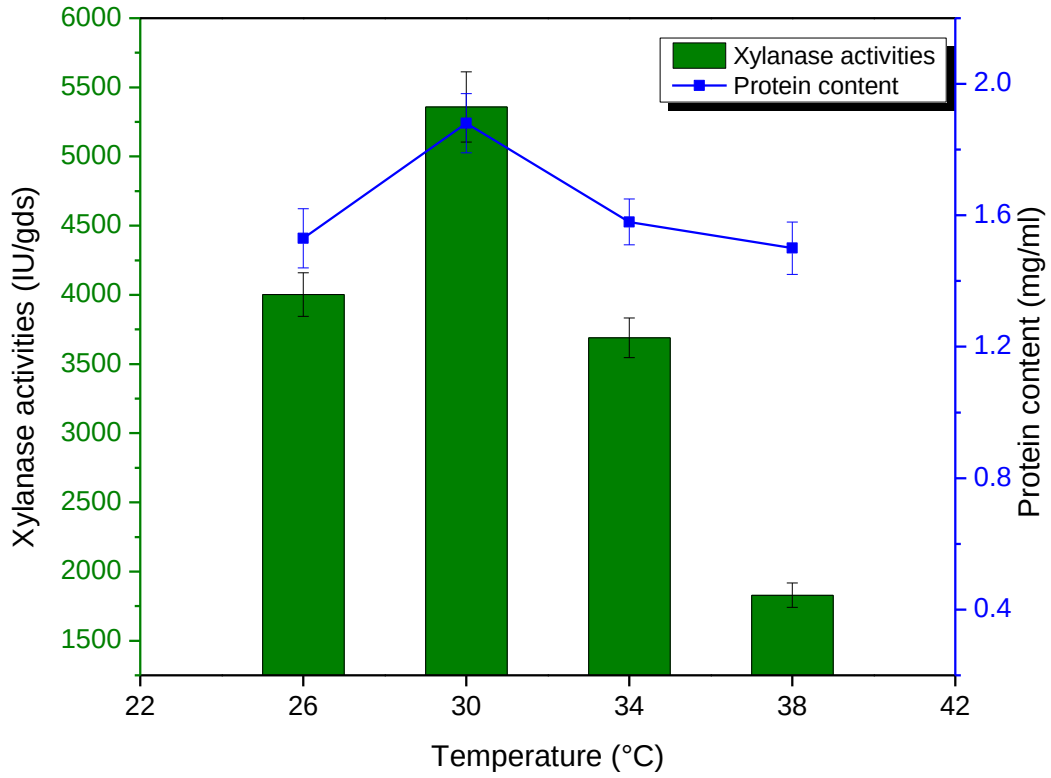

Figure-2.6: Effect of temperature on xylanase production by *S. commune* ARC-11

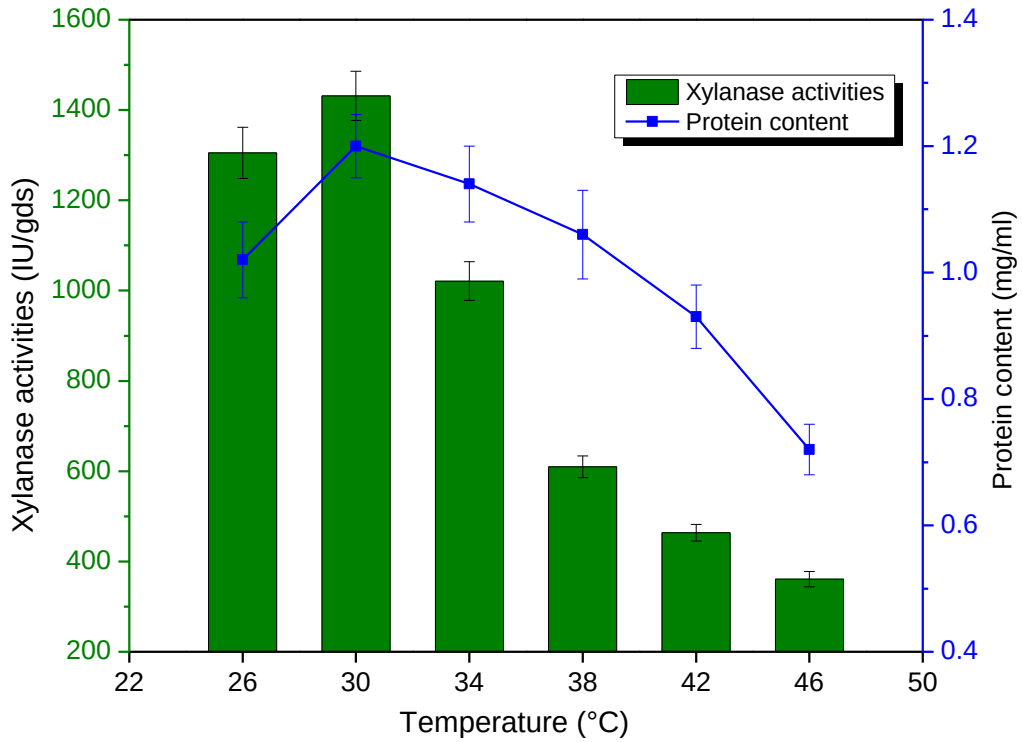

Figure 2.7: Effect of temperature on xylanase production by *A. flavus* ARC-12

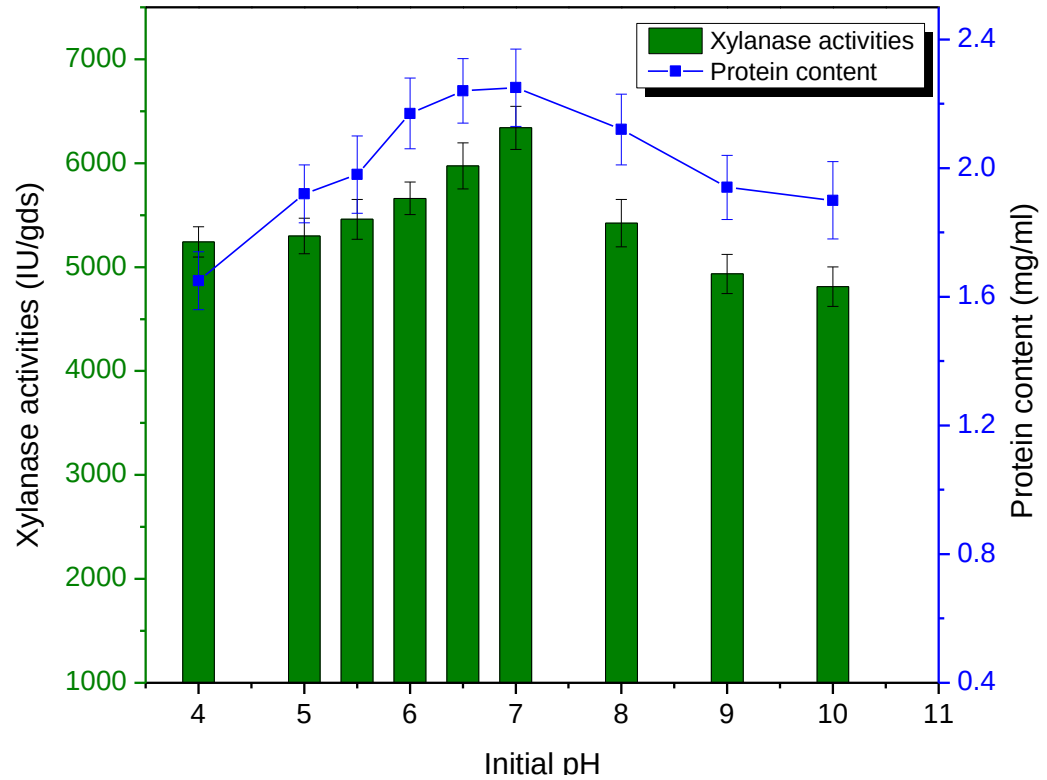

Figure-2.8: Effect of initial pH on xylanase production by *S. commune* ARC-11

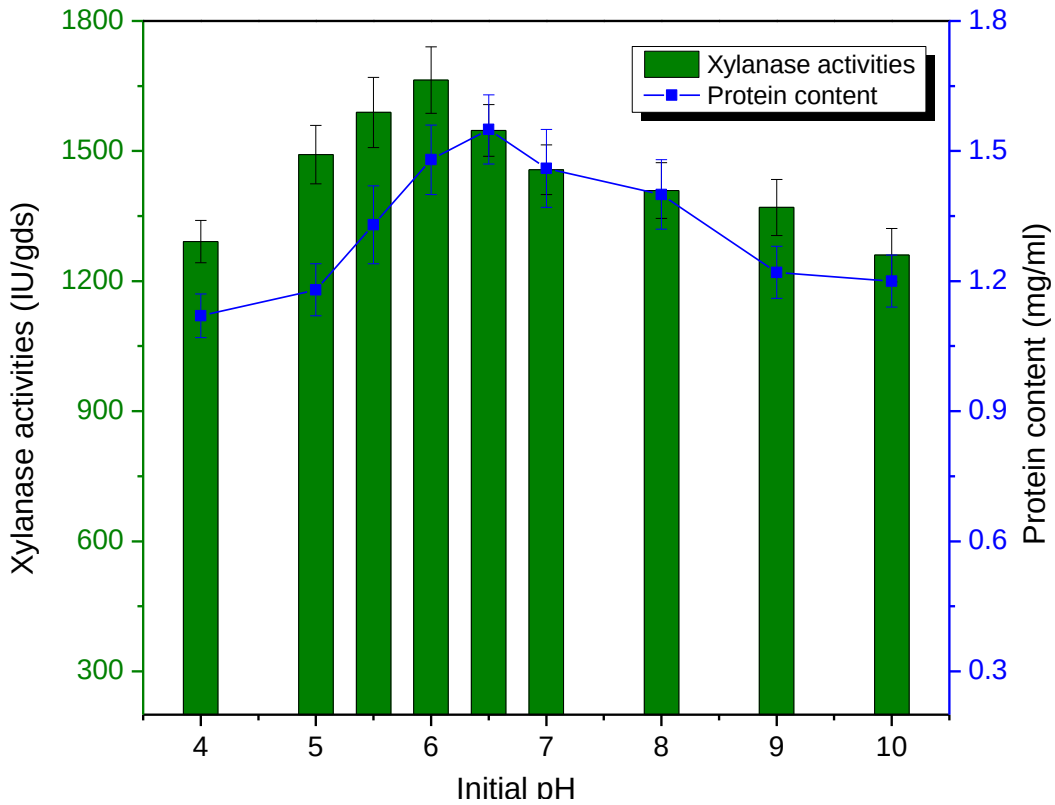

Figure-2.9: Effect of initial pH on xylanase production by *A. flavus* ARC-12

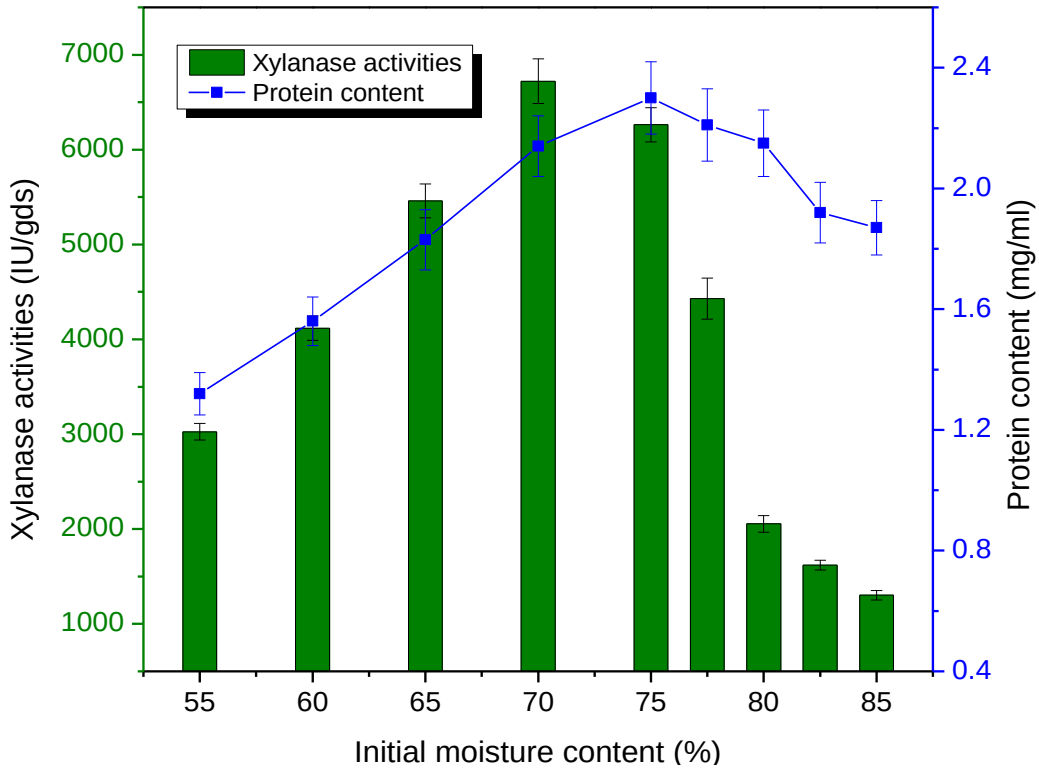

Figure-2.10: Effect of initial moisture content on xylanase production by *S. commune* ARC-11

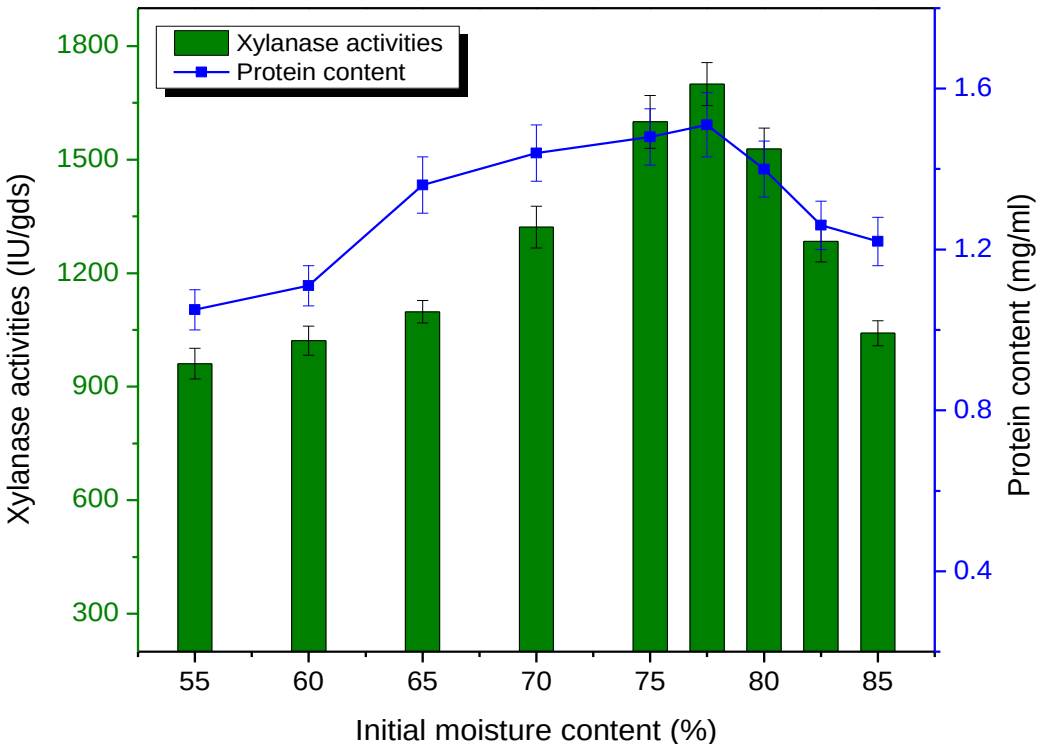

Figure-2.11: Effect of initial moisture content on xylanase production by *A. flavus* ARC-12

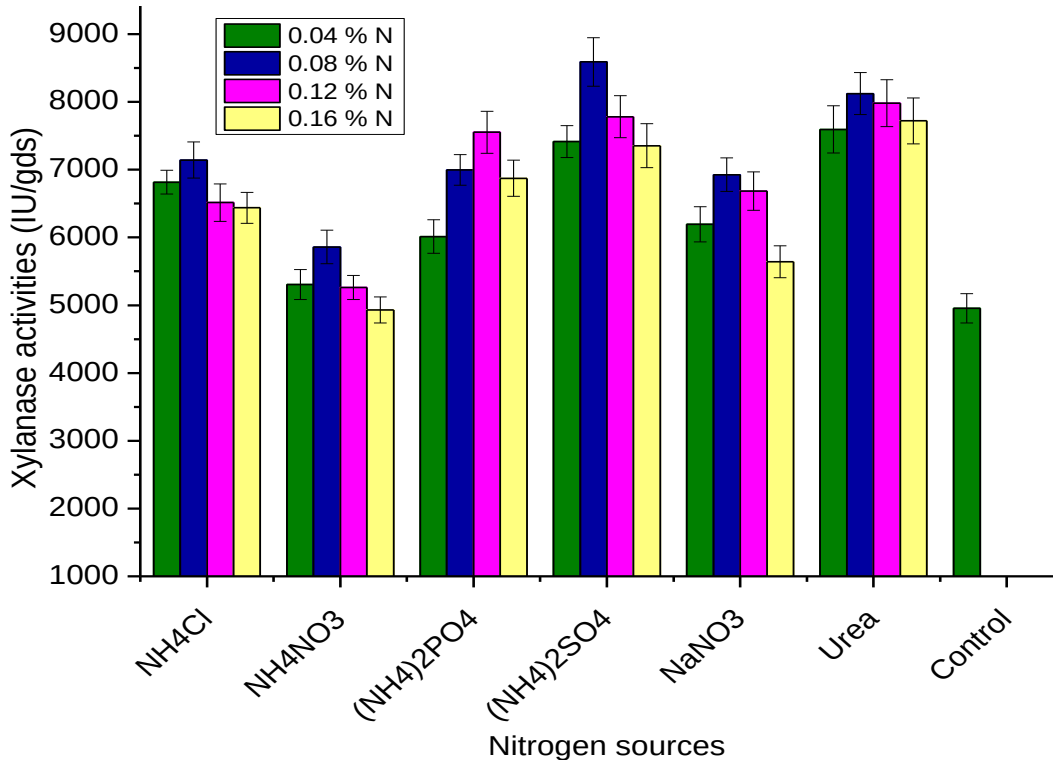

Figure-2.12: Effect of simple nitrogen sources on xylanase production by *S. commune* ARC-11

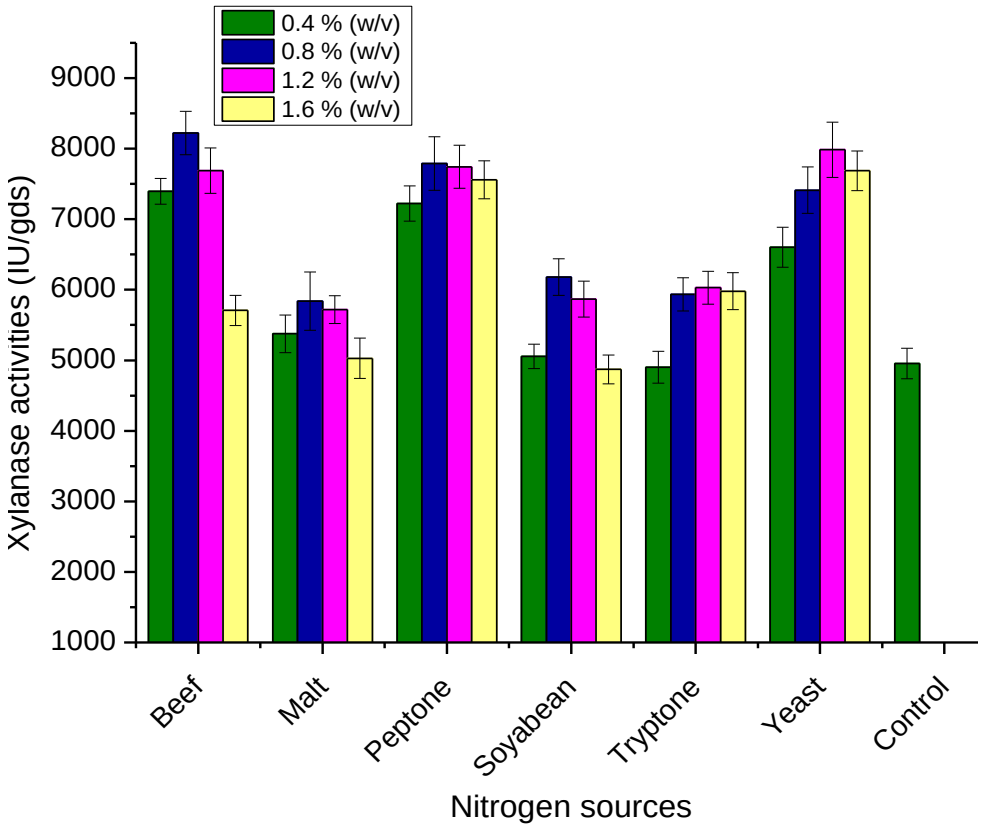

Figure-2.13: Effect of complex nitrogen sources on xylanase production by *S commune* ARC-11

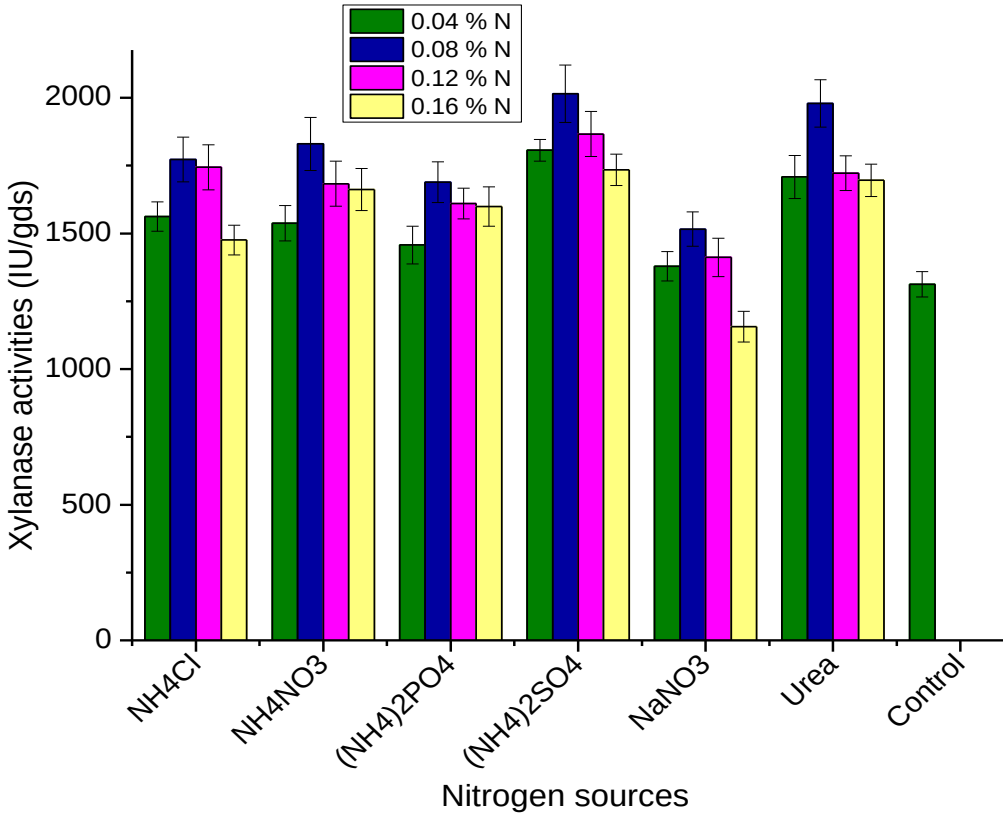

Figure-2.14: Effect of simple nitrogen sources on xylanase production by *A. flavus* ARC-12

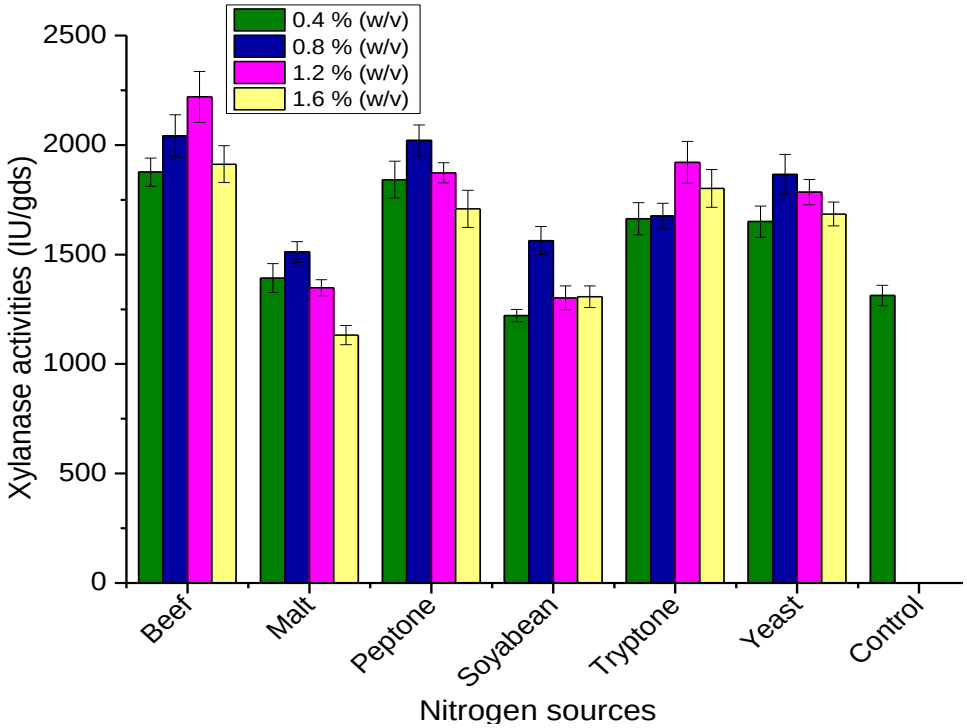

Figure-2.15: Effect of complex nitrogen sources on xylanase production by *A. flavus* ARC-12

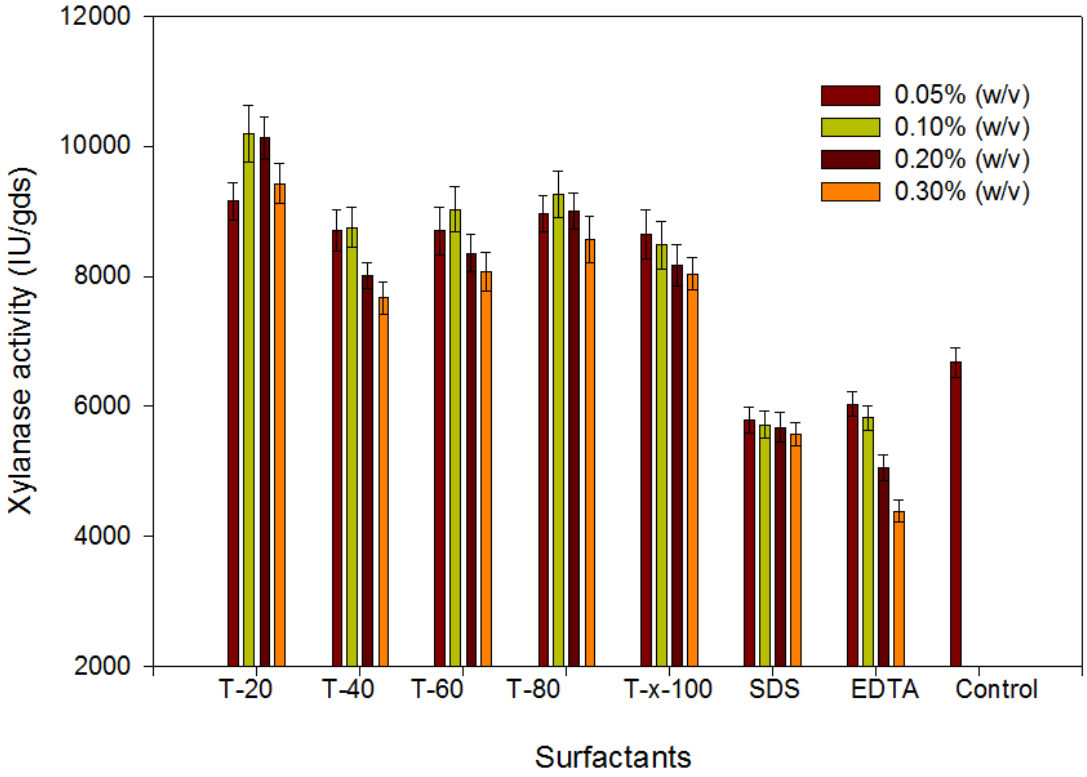

Figure-2.16: Effect of surfactants on xylanase production by *S. commune* ARC-11

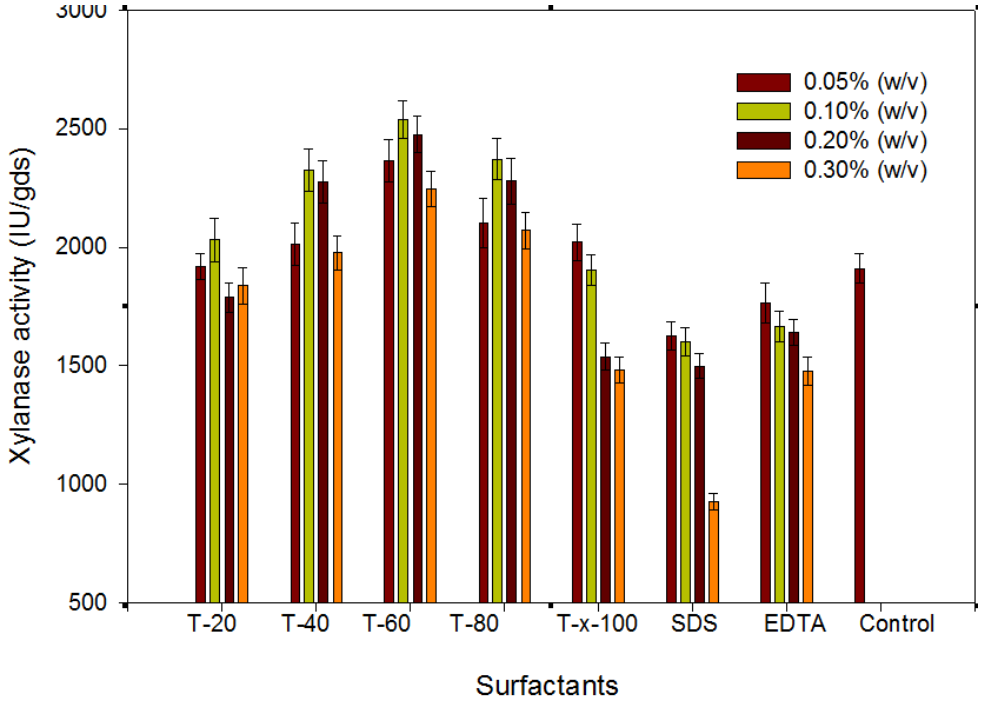

Figure-2.17: Effect of surfactants on xylanase production by *A. flavus* ARC-12

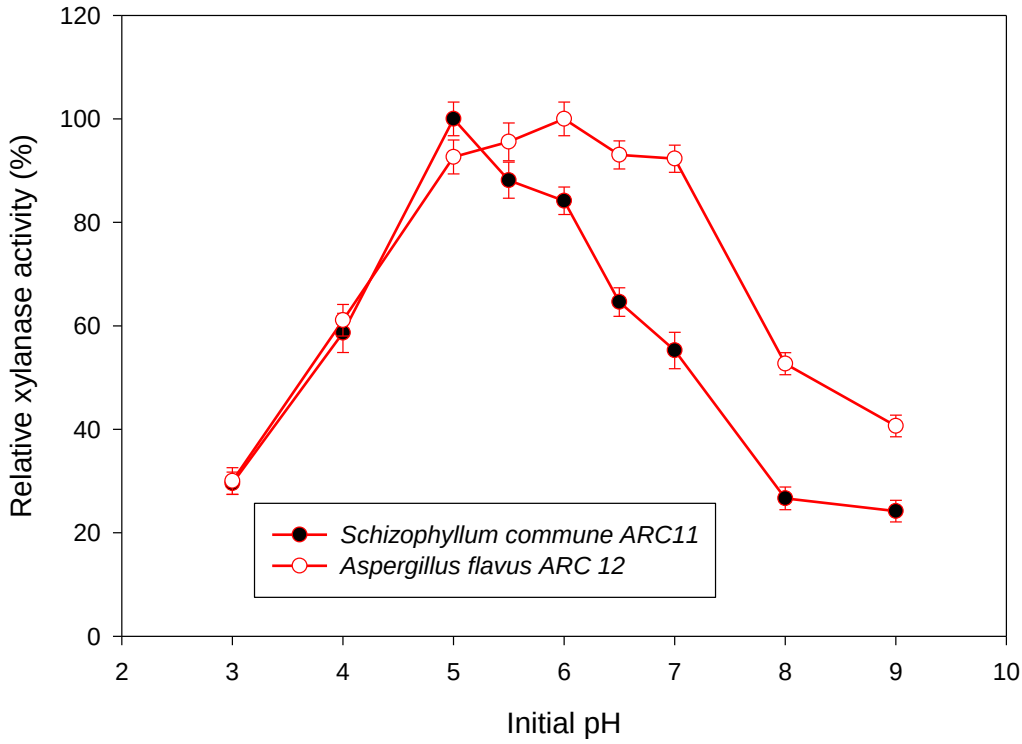

Figure-2.18: Effect of initial pH on xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12

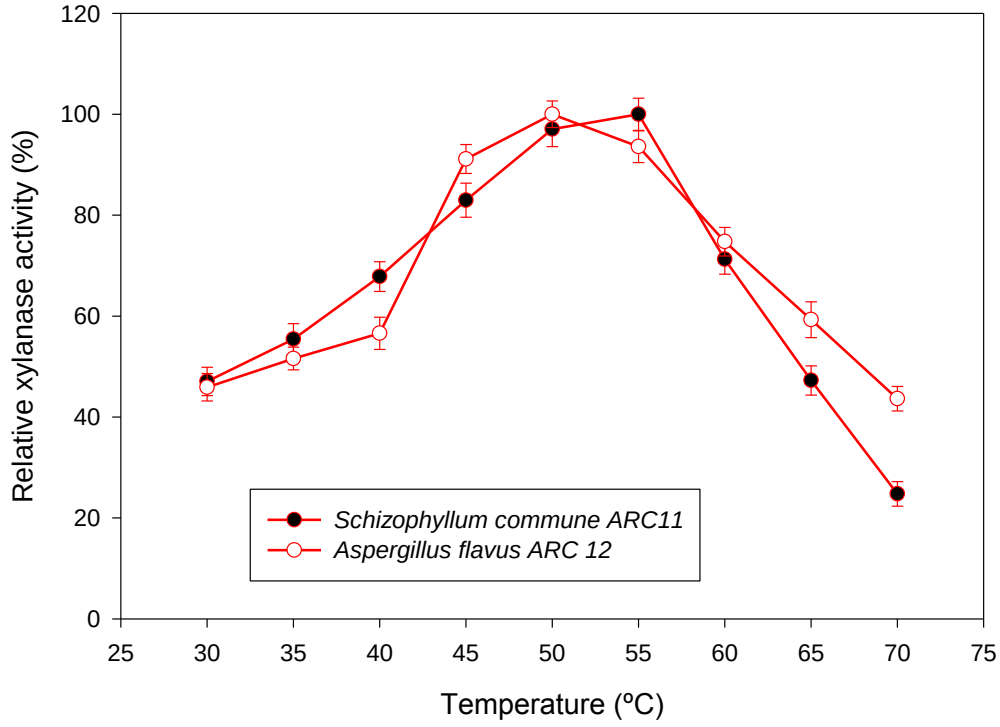

Figure-2.19: Effect of temperature on xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12

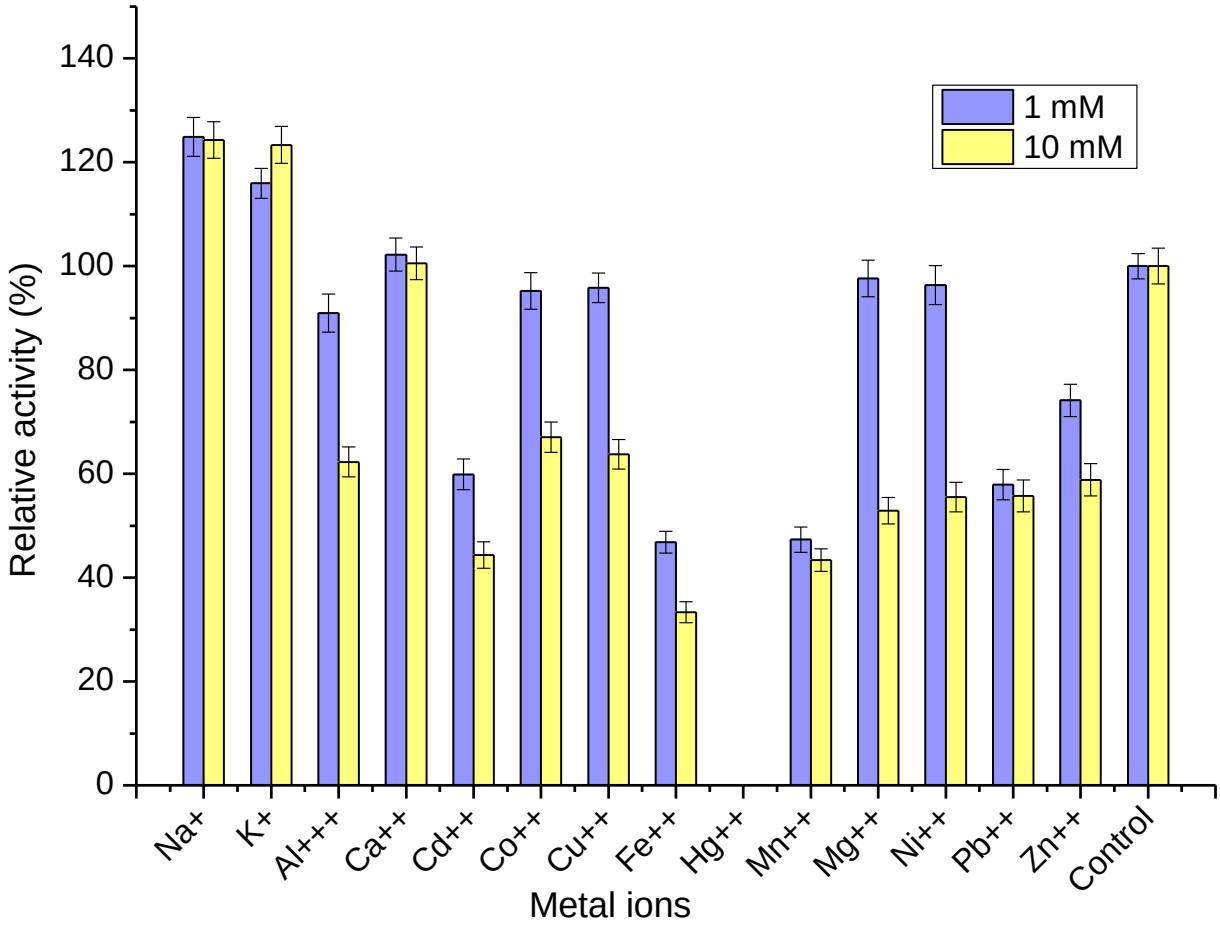

Figure-2.20: Effect of metal ions on xylanase activity from *S. commune* ARC-11

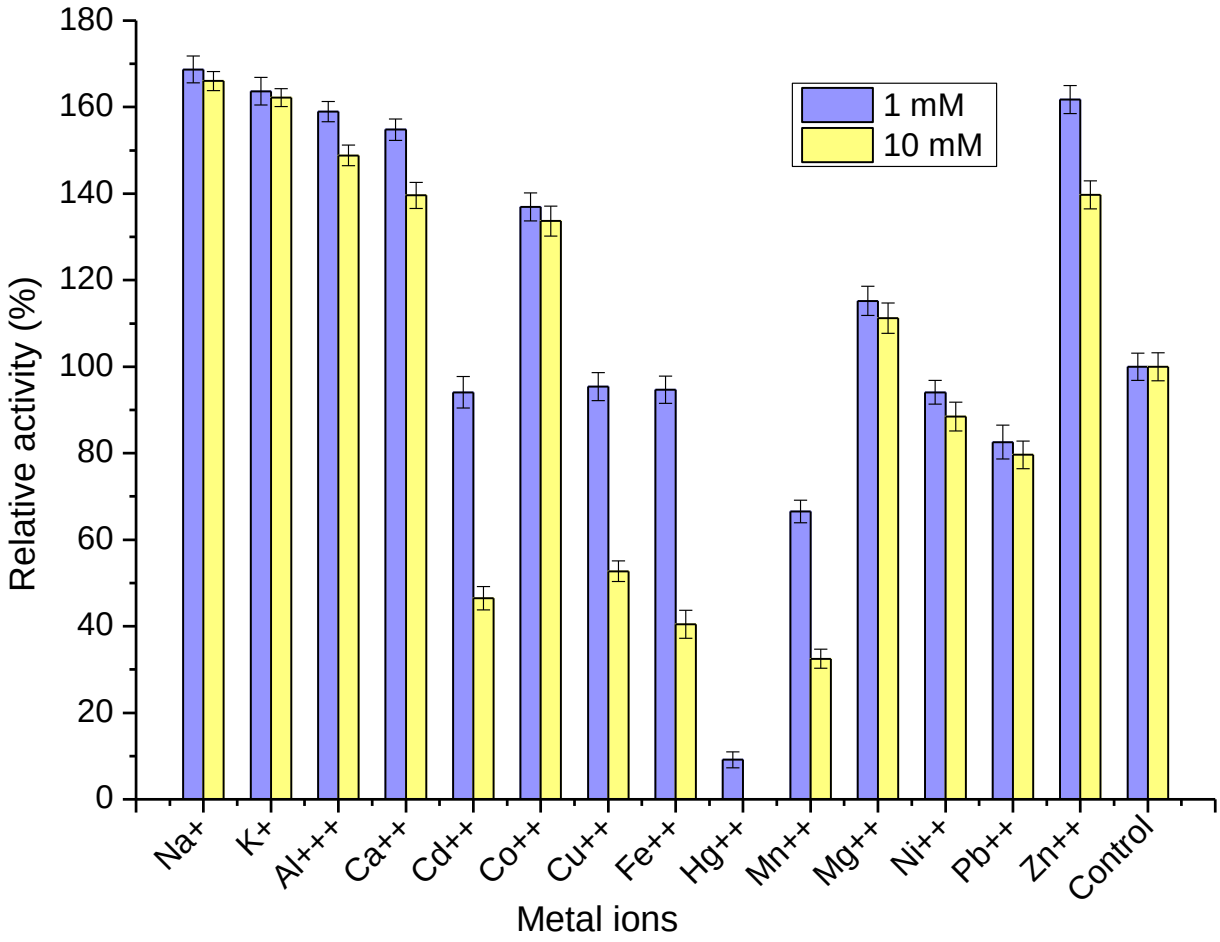

Figure-2.21: Effect of metal ions on xylanase activity from *A. flavus* ARC-12

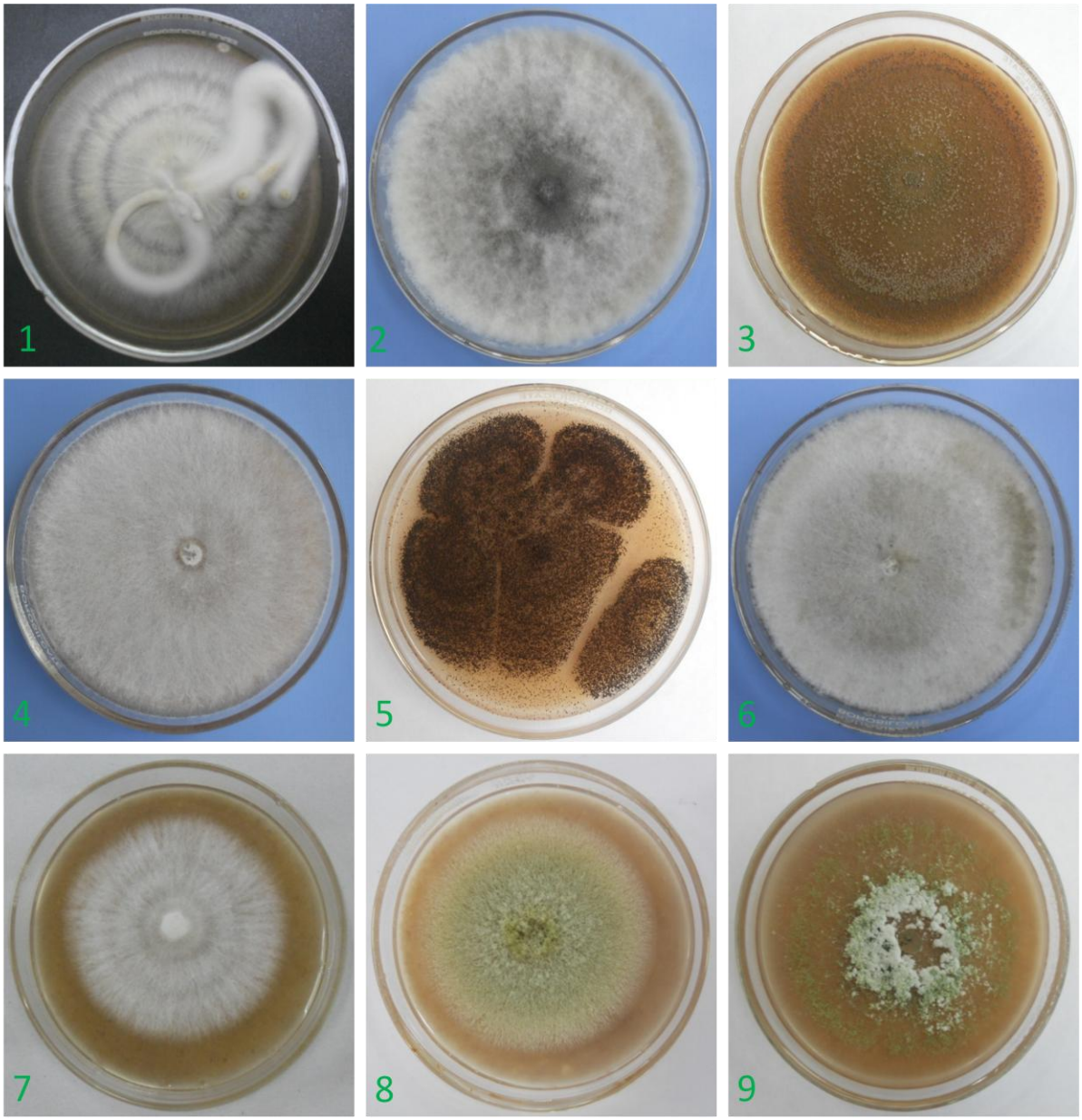

Plate-2.1: Fungal strains- 1=ARC-2, 2=ARC-5, 3=ARC-6, 4=ARC-9, 5=ARC-10, 6=ARC-16, 7=ARC-11, 8=ARC-12, 9=ARC-22

Plate-2.2: 1=bagasse, 2=congress grass, 3=corn cob, 4=maize bran, 5=millet stover, 6= sugarcane tops, 7=sabai grass, 8= wheat bran, 9=wheat straw

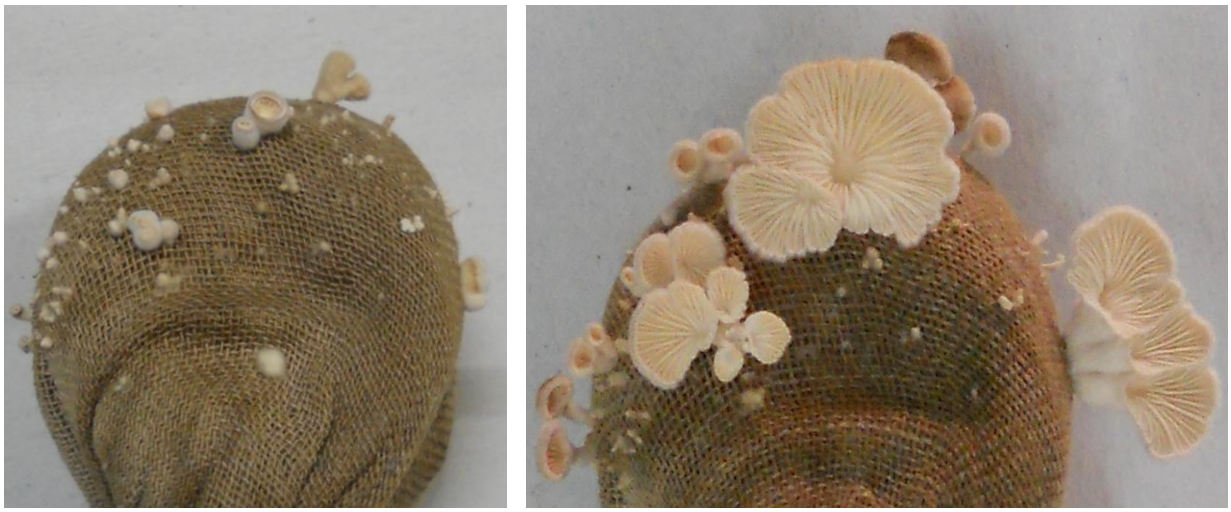

Plate-2.3: Fruiting body of *Schizophyllum commune* ARC-11: (A) after 3 days, (B) after 9 days

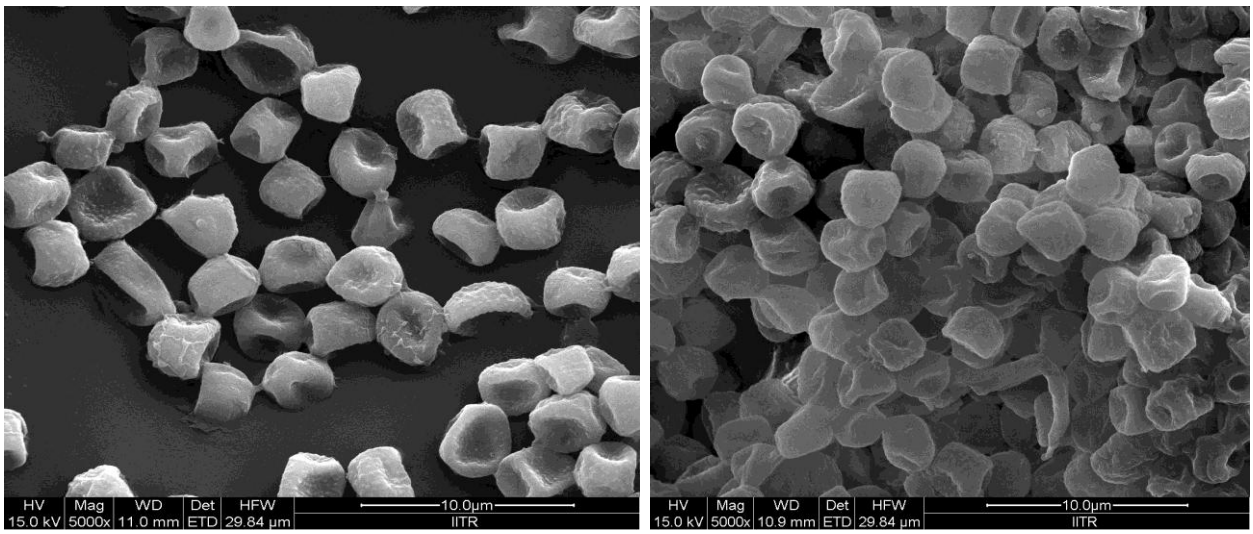

Plate-2.4: SEM images of spores of *Aspergillus flavus* ARC-12

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CHAPTER 3

MORPHOLOGICAL, CHEMICAL AND PULPING STUDIES OF *EULALIOPSIS BINATA*

3.1: Introduction

Paper is a network of cellulosic fibers which may be defined as felted sheet of fibers formed on a fine screen from a fiber-water suspension or pulp slurry. Fiber is the basic component in paper manufacturing which governs the properties of paper. Morphological characteristics of fiber play a decisive role for assessing the suitability of any raw material for pulp and paper manufacturing. The most important fiber characteristic is the fibre length which controls the strength properties of paper. Fibre length influences the tearing strength positively [1, 2]. However, longer fibers tend to give a more open and less uniform sheet structure. Fiber diameter and wall thickness governs the fiber flexibility. Paper made from thin walled fibers will be dense and well formed. Fiber lumen width affects the beating behavior of pulp [2-5]. Except fiber length, the other morphological features of fibres are fibre width, and cell wall thickness which vary significantly within the wood species, annual growth rings, different parts of stem, and growing conditions of the plant [6].

Physical strength properties of paper like tensile strength, bursting strength and folding endurance are affected mainly by the way in which individual fibres are bonded together. The bonding among fibres largely depends on fibre flexibility and compressibility [1]. Other derived morphological factors of importance are flexibility coefficient, rigidity coefficient, Runkel ratio, slenderness ratio, wall fraction and Luce's shape factor. The higher fibre flexibility increase the chance of formation of well bonded paper sheet. Likewise, in increase in fibre rigidity results in a decrease in fibre bonding. Runkel ratio and fiber to vessel ratio influences the basic density of wood [1, 2, 7]. If Runkel raio of fibre is higher, than fibres are stiffer, less flexible and forms bulkier paper with lower bonded area. The lower Runkel ratio and higher average fibre length results the good strength properties of paper [8, 9]. Runkel ratio is also related to paper conformability, pulp yield and fibre density [2]. The influence of fiber morphological properties on paper strength is summarized in Table 3.1.

Cellulose is the main component of chemical pulp and amount of cellulose in raw material directly reflects the variation in pulp yield and economy in pulp production. Paper strength also depends on cellulose content of the pulp and, mechanical strength of the pulp (especially tensile strength) is directly proportional to the cellulose content [10]. Cellulose is a long chain polymer of glucose while hemicelluloses have much lower molecular weight and degree of polymerization. During chemical pulping, hemicelluloses dissolve to a large extent along with lignin and a substantial quantity of hemicelluloses remains associated with the pulp. The content of hemicelluloses in pulp affects the swelling behavior of the pulp fiber [11]. The charge on fibre also affects the swelling of fibre. The water molecules bind the hydroxyl groups present in cellulose and hemicelluloses [6].

The need of good quality of fibre compels the paper industry to search other alternative fibre. Along with the search of new fibrous raw materials, it is also necessary to develop the environmental friendly and cost effective processes for paper making. Pulping is the process which converts wood or any other raw material into fibrous mass for paper making by breaking the bonds within substrate. The chemical pulping processes includes sulfite, kraft and soda pulping processes. Sulfite pulping process was formerly favored but now it is in a steady decline due to inferior pulp properties, inability of utilizing all types of wood and non-wood materials, and environmental concerns [12-14]. Kraft and soda pulping methods have been utilized commonly for the delignification of raw materials and production of pulp for papermaking. One major drawback for these pulping methods is lower pulp yield due low delignification selectivity [12, 15, 16]. Most of chemical pulp is produced by kraft pulping that shows better physical strength properties [12-14, 17]. In contrast, during kraft pulping process the total reduced compounds, hydrogen sulphide, and suspended solids creates environmental problem [13].

Therefore, some modified pulping processes with higher selectivity towards lignin may be more useful to obtain the high yield of pulp with better properties. Addition of organic solvent to kraft or soda pulping process enhances the selective delignification. Organic solvents such as ethanol, methanol, acetone, acetic acid, formic acid, ethylene glycol, ethanolamine etc. have been used in organosolve pulping [12, 13, 15]. The addition of solvent to soda liquor may be the important alternative to kraft pulping for eliminating the sulfur compounds. The soda pulping process is less polluting and more environmental friendly compared to other

conventional pulping methods like kraft and sulfite pulping processes. The delignification of cellulosic raw materials by soda pulping process causes the degradation of cellulose and hemicelluloses. The addition of suitable organic solvent such as ethanol enhances the selectivity of liquor towards lignin and protects carbohydrates against degradation which improves the pulp yield and strength properties [12, 13, 18, 19]. Sahin [12] studied the ethanol-soda pulping of jute and reported more selective and rapid delignification of jute, resulting marked improvement in pulp yield and strength properties. Martinez and Sanjuan [20] compared ethanol–water–soda pulping of chihuahua pine with kraft pulping process and reported that the strength properties of organosolv pulps comparable with kraft pulps. Ethanol and methanol are the frequently used organic solvents in organosolve pulping process. The behavior of ethanol and methanol during the delignification of *Eucalyptus globulus* shows better dissolution of lignin with methanol. However, at high-intensity cooking conditions ethanol pulping process produces the pulp with less lignin. The higher screened pulp yield can be obtained by the ethanol pulping process limiting the kappa number range between 20-30 units. Furthermore, the use of methanol may be hazardous due to its highly flammable and toxic nature [13, 21, 22]. The higher pulp yield with lower residual lignin, higher brightness and better strength properties can be obtained with ethanol-soda pulping process compared to soda pulping process [12, 23].

Solvents after organosolve pulping are recovered by evaporation and distillation methods. Lignin is precipitated after evaporation by decreasing the pH of the liquor and can be collected after centrifugation. The recovery system of organosolve pulping process is simpler to kraft pulping process [13, 23, 24]. During organosolve pulping, high quality of hemicelluloses and lignin degradation by-products are obtained [13, 23]. Lignin is an important by-product of pulping and sulfur-free lignin is obtained in organosolve pulping. Furthermore, low molecular weight and higher purity of lignin with higher number of reactive groups make it suitable for the production of lignin based adhesives and other products [23, 25, 26].

Bio-pulping is another approach which has been proved to be environment friendly and cost effective method for delignification of cellulosic raw materials. Pretreatment of raw material with white-rot fungi prior to pulping is called as bio-pulping. White-rot fungi produce the lignin degrading enzymes such as lignin peroxidase, laccase and manganese peroxidase

during bio-pulping process. The aim of bio-pulping is to remove or loosen lignin in wood matrix without decomposition of cellulose. During bio-pulping, lignin and hemicelluloses are removed by the action of different enzymes [27]. White rot fungi such as *Ceriporiopsis subvermispora, Phanerochates chrysosporium, Phlebiopsis gigantean, Physisporinus rivulosus, Trametes versicolor* etc. are most suitable for bio-pulping process [12, 27-30]. Bio-pulping improves the quality of pulp, properties of paper and reduces the energy costs and environmental impact compared to other traditional pulping processes [27, 31, 32].

This chapter was focused to study the anatomical and morphological characteristics of *Eulaliopsis binata* and assessed for its suitability to manufacture pulp for writing and printing grades. The effect of various cooking parameters was evaluated in terms of kappa number, screened pulp yield and rejects during soda pulping of *E. binata.* A comparison among soda, ethanol-soda and bio-soda pulping of *E. binata* was also carried out.

3.2: Materials and methods

3.2.1: Collection of raw material

Fresh *E. binata* grass was collected from Behat, Saharanpur district, located in the foothills of Shivalik Hills at the end of rainy season (Figure 1). The fresh grass was washed with water, chopped into small pieces of 4-6 cm manually. The chopped grass was dried in sunlight and stored in polythene bags for further use.

3.2.2: Morphological studies of *E. binata*

Anatomical and morphological features of *E. binata* were studied by light and scanning electron microscopy. Small slivers were obtained for fibre length determination and macerated with 10 ml of 67% HNO_3 and boiled on a water bath (100±2 °C) for 10 min. After that, the slivers were washed with distilled water and placed in small amount of distilled water. The fibre bundles were separated with a small mixer having a plastic end to avoid fibre breaking. Cross sections were cut on a Leitz base sledge microtome 1300 for fibre diameter, lumen diameter and cell was thickness determination. To enhance the visibility of cell wall, aniline sulphate-glycerine mixture (1:1) was used for staining of cross-sections of fibre. A total of 100 randomly chosen fibres were analyzed under a calibrated microscope. The derived wood properties such as flexibility coefficient [(fibre length/fibre diameter)×100], Luce's shape factor [(fibre diameter^2 - lumen diameter^2)/(fibre diameter^2 + lumen diameter^2)] [33], Runkel

ratio [(2×cell wall thickness)/ lumen diameter] [34], rigidity coefficient [2 × cell wall thickness/ fibre diameter], slenderness ratio [fibre length/fibre diameter], solid factor [(fibre diameter^2 - lumen diameter^2) × fibre length] [35] and wall fraction [(2 × cell wall thickness/ fibre diameter) ×100] were determined using fibre dimensions [2].

3.2.3: Proximate chemical analysis

For the proximate chemical analysis, *E. binata* was milled in a Wiley mill (Weverk, A-47054, Sweden) and the portion passed through –40 size mesh and retained on +80 size mesh was used for proximate analysis. The powdered fractions were subjected to water solubility (TAPPI T 207 cm-99), 1% caustic soda solubility (TAPPI T 212 om-98), and alcohol-benzene solubility (TAPPI T 204 cm-97). Extractives were removed from *E. binata* using Soxhlet apparatus and mixture of ethanol-benzene (1:2 v/v) before compositional analysis as per TAPPI test method (TAPPI T 264 cm-97). Extractive free sample of *E. binata* air-dried and subjected to chemical composition analysis such as: ash (TAPPI T 211 om-93 "Ash in wood"), α-cellulose (TAPPI T 203 cm-99 "α-, β- and γ-cellulose in pulp), holocellulose (TAPPI T 249 cm-00 "Holocellulose in wood"), lignin (TAPPI T 222 om-02 "Lignin in wood"), pentosan (TAPPI T 223 cm-01) as per TAPPI Standard Test Methods 2007 [36].

3.2.4: Pulping studies

The cooking of chopped *E. binata* was performed in an electronically heated WEVERK rotary digester of 0.02 m^3 capacity having four bombs of one liter capacity each. During soda pulping of *E. binata*, alkali dose was varied from 8 to 12% (as Na_2O) while keeping other variables constant as mentioned in Table 3.3. Similarly, the maximum cooking temperature was varied from 120 to 160 °C with an interval of 10 °C while keeping other conditions constant as shown in Table 3.4. The cooking time was varied from 60 to 210 min with an interval of 30 min keeping other conditions constant as in Table 3.5. The biomass to moisture ratio was varied from 1:1 to 1:5 at optimized cooking conditions. After optimizing the parameters associated with soda pulping, the effect of ethanol to soda pulping process at concentration ranging from 20 to 35% (w/v) was also tested.

Biological pretreatment of *E. binata* was carried out with a white-rot fungus *Schizophyllum commune* ARC-12. During fungal pretreatment, initial moisture content of was adjusted to 70% and incubated at 30 °C for 12 days. After pretreatment, the pulping of *E.*

binata was carried out at optimum cooking conditions. After completion of *E. binata* digestion, residual cooking chemicals were removed by washing with tap water on a laboratory flat stationary screen of 300 size mesh. The washed pulp was disintegrated and screened in a laboratory Weverk vibratory flat screen having a slot size of 0.15 mm. The screened pulp was washed with tap water, pressed, crumbled and air-dried. All the pulp samples were evaluated for screened pulp yield, rejects and kappa number (TAPPI T 236 cm-85 "Kappa number of pulp") according to TAPPI Test Standards 2007 [36]. Reducing sugars in black liquor were determined as per DNS method (Miller 1959) [37].

3.2.5: Pulp beating, laboratory handsheets preparation and testing

Unbleached pulp samples were beaten in a PFI mill (TAPPI T 248 sp-00 "Laboratory beating of pulp") at fixed beating level of 35 °SR. Laboratory handsheets of 60 g/m^2 were prepared using British sheet former (TAPPI T 205 sp-2 "Forming handsheets for physical tests of pulp"). The handsheets were preconditioned at a temperature of 27±2 °C and relative humidity of 65±2% and evaluated for physical strength properties such as burst index (TAPPI T-403 om-02 "Bursting strength of paper"), tensile index (TAPPI T-404 wd-03 "Tensile breaking strength and elongation of paper and paperboard"), double fold (TAPPI T-423 cm-98 "Folding endurance of paper") and tear index (TAPPI T 414 om-04 "Internal tearing resistance of paper"). Thick pads of 4±0.2 g were prepared (TAPPI T 218 sp-02 "Forming handsheets for reflectance testing of pulp" (Büchner funnel procedure)) of unbleached soda, ethanol soda and bio-soda pulps for brightness determination according to TAPPI Test Standard TAPPI T 452 om-02 (Brightness of pulp, paper, and paperboard (directional reflectance at 457 nm)). All the pulps were also tested for viscosity according to TAPPI Test standards (TAPPI T 230 om-04 "Viscosity of pulp (capillary viscometer method)) [36].

3.2.6: FE-SEM analysis

FE-SEM analysis was carried out to study the anatomical features of *E. binata*. Morphological studies of soda, ethanol-soda and bio-soda pulps were carried out by FE-SEM (Leo 435 VP, England). The cross-sections of *E. binata* grass were subjected to fixation by using 3% glutaraldehyde (v/v) and 2% formaldehyde (4:1) for 6 h. The samples were washed thrice with distilled water after primary fixation and treated with ethanol gradients of 30-90 with a difference of 10% and absolute ethyl alcohol (99.9%) for dehydration. The samples were

kept for 15 min in 30 to 90% ethanol and for 30 min in absolute ethyl alcohol. After dehydration, samples were air-dried and examined under FE-SEM. Before injecting the sample in a sample chamber, samples were coated with gold by a standard sputtering technique for 30 s. Electron photomicrographs were taken at suitable voltage and magnifications.

3.2.7: XRD analysis of pulp samples

XRD analysis was carried out to determine the crystallinities of soda, ethanol-soda and bio-soda pulps by a Ultima IV Rigaku X-Ray Diffractometer using Cu Kα radiation (λ=1.5405 Å) at 40 kV and 40 mA. Samples were scanned at angle 2θ ranging from 5 to 60º with a speed of 2º/ min^{-1} and a step size of 0.02º. Crystallinity index was calculated as a ratio between the area of the crystalline contribution and total area by using XRD amorphous subtraction method [38]

3.2.8: Statistical analysis

All the experiments were carried out in triplicate and experimental results were represented as the mean ± standard deviation of values.

3.3: Results and discussion

3.3.1: Morphological studies of *E. binata*

The role of morphological characteristics and their derived values such as flexibility coefficient, slenderness ratio, rigidity coefficient, wall fraction, Runkel ratio and Luce's shape factor of *E. binata* [2, 3] and reported in Table 3.1. Fibre length for *E. binata* was 2.20 mm which was higher compared to other grasses such as bamboo (1.91 mm), lemon grass (1.09 mm) and sofia grass (0.87 mm). Fibre diameter for *E. binata* (10.85 µm) was less than bamboo (16.8 µm), lemon grass (16.3 µm) and sofia grass (14.7 µm) respectively. A higher fibre length showed higher tearing strength of paper [2, 8]. The fibre diameter and cell wall thickness controlled the fibre flexibility. The thickness of cell wall affects most of the paper properties such as tensile strength, burst strength and folding endurance. The paper made of thick-walled fibres has low tensile strength, burst strength and folding endurance. The laboratory handsheets would be bulky, coarse-surfaced and had higher void volume. Paper formed by thin-walled fibre would be dense, and well formed [2, 8]. *E. binata* showed higher slenderness ratio (202.76) compared to bamboo (114), lemon grass (66.9) and sofia grass (59.2). Slenderness ratio (fibre length/fibre diameter) affects the paper properties positively. Generally, it is

considered that if the slenderness ratio for the fibre is less than 70 than pulp would have poor strength [8, 9]. Lumen diameter for *E. binata* fibre was 5.86 µm which was less than lemon grass (6.73 µm) and higher than sofia grass (5.07 µm) and bamboo (3.31 µm). Lumen diameter is an important parameter during pulp beating; a higher lumen diameter facilitates the penetration of liquid during pulp beating [2]. The Runkel ratio of *E. binata* (1.41) was less than bamboo (4.08), lemon (1.45) and sofia grasses (1.52). If Runkel ratio of fibre is higher, than fibres are stiffer, less flexible and forms bulkier paper with lower bonded area. The lower Runkel ratio and higher average fibre length results the good strength properties of paper [8, 9]. Runkel ratio was also related to paper conformability, pulp yield and fibre density [2]. Luce's shape factor of *E. binata* (0.54) was less than lemon grass (0.71) and sofia grass (0.79). Luce's shape factor and solid factor are related to paper sheet density and could be significantly correlated to breaking length of paper [39]. Solid factor of *E. binata* was 183.45 less than lemon (240.24) and higher than sofia grass (165.63). Burst strength and breaking length are determined by collapsibility of fibres to double walled ribbons on pressing. Thick walled, narrow lumen and higher fibre length is attributed to maximum solid factor.

3.3.2: Proximate chemical analysis of *E. binata*

Cold water solubility of *E. binata* is more than bamboo and less than lemon and sofia grasses while hot water solubility for *E. binata* is higher than bamboo and sofia grass and lower than lemon grass. The cold water treatment removes a part of extraneous components like tannins, gums, sugars, inorganic matter and colored compounds present in lignocellulosic biomass whereas hot water treatment removes, in addition, starches. The higher water solubility adversely affects the pulp yield [3]. Ethanol-benzene solubility of *E. binata* was 3.9% compared to bamboo (2.3%), lemon grass (4.3%) and sofia grass (5.9%). Ethanol-benzene extractives include waxes, fats, resins, low-molecular weight carbohydrates, photo-sterols, non-volatile hydrocarbons, salts and other water-soluble substances. Ethanol-benzene extractable content precipitates and adversely affects the runnability of process equipment due to blocking of openings in Fourdrinier wire. It also affects the quality of paper because of shadow marking and paper manufactured from such type of fibrous material might show reduced water absorbency [3]. 1% NaOH solubility of *E. binata* (38%) was higher compared to bamboo (24.7%), lemon grass (30.6%) and sofia grass (28.2%) while it was lower compared to rice straw (57.7%), and sunflower stalks (50.4%) [40, 41]. The higher NaOH solubility of *E.*

binata was possibility due to the presence of low molar mass of carbohydrates and other alkali soluble materials. Holocellulose and α-cellulose contents were 73.1% and 46.0% in *E. binata* which were comparable to bamboo, lemon grass and sofia grass (Table 3.2). Lignocellulosic materials with 34% or higher cellulose content are regarded as pulp and paper production from a chemical composition point of view [42, 43]. Holocellulose and α-cellulose contents in plant biomass positively influence the yield of pulp during chemical pulping methods. The cellulose content of cellulosic raw materials also determines physical strength properties of paper. [43]. Lignin content of *E. binata* was 21.2% compared to bamboo (24.7%), lemon grass (17.4%) and sofia grass (17.0%). Lignin is undesirable polymer for paper production and the removal of lignin during pulping requires the high amount of energy and chemicals. Lower lignin content of raw materials makes them suitable for delignification at milder pulping conditions (lower temperatures and chemical charges) to reach a desirable kappa number [43, 44]. Ash content of *E. binata* was found lower compared to lemon grass and higher than bamboo and sofia grass. The mineral components of lignocellulosic biomass represented as ash content. Higher ash content is undesirable during refining and recovery of cooking liquor. It is well established that transition metals such as Mn, Fe and Cu negatively affects pulp bleachability (hydrogen peroxide and oxygen) and bleaching selectivity [8, 44].

3.3.3: Effect of cooking parameters on pulp yield, rejects and kappa number

Morphological characteristics and proximate chemical analysis of *E. binata* indicated that, the raw material has the potential to produce good quality of pulp with low active alkali and milder cooking conditions. Lower lignin content and higher 1% NaOH solubility of the material indicated that low active alkali may results satisfactory separation of cell wall to produce the pulp of acceptable quality. FE-SEM analysis of *E. binata* revealed the loose and open anatomy of substrate which facilitates penetration the cooking liquor throughout the raw material (Figure 3.2).

Soda pulping of *E. binata* was carried out using 8 to 16% of active alkali (as Na_2O) while keeping other conditions constant like pulping temperature, pulping time and bath ratio. The screened pulp yield increased up to 12% of active alkali (as Na_2O) and declined thereafter. Maximum screened pulp yield (40.21%) of kappa number 17.25 was obtained at an alkali dose of 12% (as Na_2O) (Figure 3.3). Kappa number and screening rejects declined sharply up to 12% of active alkali charge and became almost constant thereafter. 12% of active alkali was

found optimum for the delignification of *E. binata* (Table 3.3). Active alkali is one of the major factors which affect the degree of delignification and breaking down of carbohydrates significantly in the process of soda pulping. Another set of experiments showed that *E. binata* produced screened pulp yield of kappa number and screening rejects by 36.76%, 23.23 and 6.34% respectively at a pulping temperature of 120 °C (Table 3.4). Further, increase in temperature from 120 to 130 °C, screening rejects reduced to 0.9% and screened pulp yield improved to 41.12% (Figure 3.4). Beyond a cooking temperature of 130 °C, screened pulp yield decreased while delignification increased slightly. The temperature of 130 °C was found optimum to produce maximum pulp yield with acceptable kappa number. The dissolution of lignin and cellulose accelerated by increasing the temperature [18]. Figure 3.5 shows the effect of cooking time on delignification of *E. binata* during soda pulping, while keeping other variables constant. The maximum screened pulp yield (42.36%) with kappa number (17.27) was obtained at cooking time of 120 min and further increase in cooking time decreased the screened pulp yield significantly while change in kappa number was insignificant (Table 3.5). Therefore, an optimum cooking time for soda pulping of *E. binata* was 120 min. The increase in solid to liquor ratio 1:1 to 1:4 at optimum pulping conditions improved the screened pulp yield from 38.57 to 43.58% while kappa number dropped from 24.74 to 17.38 units. Further increase in solid to liquor ratio adversely affected the screened pulp yield (Figure 3.6). Finally, it was concluded that maximum pulp yield (43.58%) of kappa number 17.38 with 0.9% screening rejects was obtained at 12% of active alkali (as Na_2O) pulping temperature 140 °C, cooking time 120 min and solid to liquor ratio 1:4 (Table 3.6). Kaur et al. [45] studied the soda pulping of Sofia and lemon grass reported a maximum pulp yield of 43.5% during soda pulping of Sofia grass with 14% active alkali (as Na_2O) at pulping temperature 160 °C and pulping time 90 min. Likewise, a maximum pulp yield of 41.4% was obtained during soda pulping of lemon grass at the similar conditions except temperature (150 °C).

3.3.4: Ethanol-soda and bio-soda pulping of *E. binata*

E. binata was delignified by ethanol-soda and bio-soda pulping processes and compared with soda pulping process in terms of screened pulp yield, kappa number, brightness and physical strength properties. Ethanol was mixed with soda liquor varying the doses from 20 to 35 % (v/w) with a gap of 5% and delignified as per optimum conditions maintained during soda pulping of *E. binata*. The maximum pulp yield of 47.48% with a kappa number of 16.13

was obtained using 30% ethanol during soda pulping. The pulp yield was improved by 3.9 and 4.72% compared to soda and bio-soda pulping processes respectively while kappa number reduced by 1.25 units compared to soda pulping process and bio-soda pulping process did not show any significant reduction in kappa number (Table 3.7). It is well established that cleavage of α-O-4 and β-O-4 linkages in the lignin is necessary for lignin dissolution during pulping. After cleaving of these linkages, lower molecular weight and solvent soluble fragments of lignin are formed. Addition of ethanol to soda cooking liquor may cause improvement in the solubility of lignin in the liquor. Depolymerized lignin fragments that are larger than pore size of substrate cell wall solubilized in cooking liquor and removal occurred through cell wall [12, 15]. Hilder-brand's solubility of the solvent is an important parameter for the polymer solubility. A solvent should have the solubility parameter close to 11 (as much as possible) for higher solubility of lignin and the solubility parameter of ethanol is 12.7 whereas solubility parameter for water is 23.4 [15, 46, 47]. Soda pulping showed the higher yield losses due to degradation of carbohydrates and less selective delignification. On the other hand, addition of ethanol to soda liquor improved the selective delignification [12, 15]. The addition of ethanol to soda liquor reduced the dissolving power of liquor which protected the cellulosic fibre against degradation, thereby improving the pulp yield [12, 18, 23]. Several researchers have reported improvement in pulp yield up to 10% by ethanol-soda pulping compared to soda pulping [12, 18, 48]. The amount of reducing sugars released in black liquor during soda pulping (2.27 mg/ml) was higher compared to 1.92 and 1.62 mg/ml for bio-soda and ethanol-soda pulping of *E. binata* respectively. Crystallinity index of ethanol-soda was 46.21% compared to 43.39 and 42.54% for soda and bio-soda pulps respectively (Figure 3.8). During soda pulping cellulose and hemicelluloses undergoes peeling reactions in which single monosaccharide units sequentially are removed from the reducing end of carbohydrate chain. The higher crystallinity index in ethanol-soda pulping can be due to the fact that ethanol protects the carbohydrates against reactions during cooking processes and recrystallization of amorphous glucan occurred concurrently during pulping [12, 48]. The results of viscosity also validated the increased pulp yield during ethanol-soda pulping of *E. binata*. Ethanol-soda pulp showed maximum pulp viscosity (29.22 cps) compared to 23.16 and 21.54 cps for bio-soda and soda pulps respectively (Table 3.8). Similar findings were also reported by Akgul et al. [49], who observed 14.4 and 17% increase in viscosity during with the addition of 40 and 50% of ethanol to soda pulping of cotton stalk compared to soda pulping. Pulp yield of ethanol-soda of

cotton stalk was also increased by 13.5 and 14% with the addition of 40 and 50% of ethanol to soda pulping [49]. Gumuskaya et al. [48] reported an increase of 9.85% in pulp yield and during ethanol-soda pulping of cotton linters at 160 °C compared to soda pulping at the same cooking conditions. Higher pulp viscosity of ethanol pulp with higher pulp yield was also reported by Sridach [13].

Pulp brightness (ISO) after ethanol-soda and bio-soda pulping of *E. binata* was improved by 6.6 and 4.1% respectively compared to soda pulping. The improvement in pulp brightness was due to selective removal of lignin fragments during ethanol-soda and bio-soda pulping processes. A comparison among physical strength properties were done for all the three types of pulps at a fixed beating level of 35±1 °SR. An improvement in physical strength properties was observed in case of ethanol-soda and bio-soda compared to soda pulping. Addition of 30% ethanol during soda pulping of *E. binata*, improved the pulp brightness by 6.6%, tensile index 32.18%, burst index 35.40% and double fold numbers 77.31% compared to soda pulping (Table 3.9). On contrary to this, tear index of ethanol-soda pulp decreased by 9.95% compared to soda pulp. Similarly, bio-soda pulp showed an improvement in tensile index, burst index and double fold numbers by 24.94, 48.45 and 14.03% respectively compared to soda pulp. Following the same pattern, tear index of bio-soda pulp decreased by 12.86% compared to soda pulping. SEM analysis showed higher bonding among the fibres of ethanol-soda and bio-soda pulps compared to soda pulp (Figure 3.7). The bonding of fibres is an important factor for paper properties. The bonding among fibres depends on hydrophilic nature of fibre surface, and consequently on hydrogen bond formation ability of fibres. The presence of hemicelluloses favors the hydrogen bond formation ability and bonding of fibres which in turn improves the paper properties. The hydrophobic nature of lignin in fibre may directly affect the properties of paper. During delignification by soda pulping, lignin condensation and precipitation on fibre surface occurs which may affect the hydrogen bonding of fibres. Moreover, pulps with higher lignin content show slow beating and poor inter-fibre bonding which results into low sheet density and inferior strength properties [12, 50, 51]. In ethanol-soda pulping, the selective lignin removal and retention of hemicelluloses and less degradation of cellulose chains resulted into superior physical strength properties of paper. The fibre strength and degree of bonding between fibres govern the tensile strength of paper. Tear strength depends upon fibre length as well as on fibre boding. The tear strength starts to

decline due to an increase in bonding strength beyond a certain level [2, 12, 50, 51]. The necessary work that has to be done to pull the fibers loose depends on the length of the fibers as well as the bond strength. At higher levels of beating the inter fiber bond strength will be higher and fibers start to break instead of being pulled out intact. It takes less work to break a fiber than to pull it out (at least for long fibers) and the tear strength goes through a maximum as bond strength increases. The higher strength properties for ethanol-soda pulp compared to soda pulp have been reported by various researchers [12, 13]. Sahin [12] observed 34.88 and 11.84% improvement in burst and tensile strength after ethanol-soda pulping of jute compared to soda pulping. Akgul and Tozluolu [49] reported, 56.52 and 44.71% enhancement in burst and tensile index of ethanol-soda pulp respectively (40% ethanol and 18% NaOH) compared to soda pulp delignified at 18% active alkali and at a fixed beaten time of one min.

Table-3.1: Morphological characteristics of *E. binata*

Particulars	***E.* binata**	**Bamboo [3]**	**Lemon grass [2]**	**Sofia grass [2]**
Fibre length, (L) mm	2.20	1.91	1.09±0.43	0.87
Fibre width, (D) µm	10.85	16.8	16.3±1.6	14.7
Lumen diameter (d), µm	5.86	3.31	6.73±0.4	5.07
Cell wall thickness (w), µm	4.14	6.75	4.62±0.2	3.86
Flexibility coefficient [(d/D)×100]	54.00	19.70	31.1	30.0
Slenderness ratio (L/D)	202.76	114	66.9	59.2
Rigidity coefficient (2w/D)	0.76	0.80	0.57	0.53
Wall fraction (2w/D) ×100	76	80	57	53
Runkel ratio (2w/d)	1.41	4.08	1.45	1.52
Luce's shape factor [$(D^2-d^2)/(D^2+d^2)$]	0.54	0.92	0.71	0.79
Solid factor $(D^2-d^2)\times L$	183.45	-	240.24	165.63

Table-3.2: Proximate chemical analysis of *Eulaliopsis binata*

S. No.	**Particulars (%)**	***E.* binata**	**Bamboo [3]**	**Lemon grass [2]**	**Sofia grass [2]**
1	Cold water solubility	5.8	3.7	10.9	8.6
2	Hot water solubility	9.3	6.7	12.1	7.4
3	NaOH solubility	38.0	24.7	30.6	28.2
4	Ethanol-benzene solubility	3.9	2.3	4.3	5.9
5	*Holocellulose	73.1	73.8	72.1	72.2
6	*α-Cellulose	46.0	45.1	44.2	45.6
7	*Pentosan	21.4	16.0	25.6	21.9
8	*$^{※}$Lignin	21.2	24.7	17.4	17.0
9	Ash content	6.1	2.6	7.05	5.1

**Note: ***Extractive free basis, $^{※}$ Corrected for ash

Table-3.3: Effect of alkali dose on pulp yield, kappa number and rejects

Alkali dose (%)	Unscreened pulp yield	Screened pulp yield (%)	Kappa no.	Rejects (%)
8	44.72	36.42±1.14	37.52±1.78	8.30±0.18
10	43.39	38.50±1.06	24.90±1.02	4.89±0.12
12	**40.96**	**40.21±0.82**	**17.25±0.51**	**0.75±0.08**
14	37.75	37.23±0.96	14.20±0.73	0.52±0.06
16	35.45	35.04±0.85	12.14±0.52	0.41±0.07

Pulping conditions:

Time from ambient temperature to 105 ^{0}C = 45 min
Time from 105 ^{0}C to 150 ^{0}C = 45 min
Time at 150 ^{0}C = 150 min
Bath ratio = 1:5
Temperature = 150 °C
Alkali dose = Varied (8-12%, as Na_2O)

Table-3.4: Effect of temperature on pulp yield, kappa number and rejects

Temperature (°C)	Unscreened pulp yield	Screened pulp yield (%)	Kappa no.	Rejects (%)
110				
120	43.10	36.76±1.16	23.23±0.31	6.34±0.13
130	**42.00**	**41.12±0.56**	**17.91±0.58**	**0.88±0.04**
140	41.50	40.74±0.58	17.56±0.41	0.76±0.03
150	40.82	40.10±0.82	17.33±0.36	0.72±0.02
160	39.50	38.85±0.52	16.12±0.48	0.65±0.02

Pulping conditions:

Time from ambient temperature to 105 $^\circ$C = 45 min
Time from 105 ^{0}C to 135 ^{0}C = 35 min
Time at 130 ^{0}C = 150 min
Bath ratio = 1:5
Temperature = Varied (110-150 °C)
Alkali dose = 12% (as Na_2O)

Table-3.5: Effect of cooking time on pulp yield, kappa number and rejects

Cooking time (min)	Unscreened pulp yield (%)	Screened pulp yield (%)	Kappa no.	Rejects (%)
60	44.49	39.17±1.24	26.52±1.34	5.32±0.18
90	43.59	41.14±0.75	19.16±0.79	2.45±0.10
120	**43.18**	**42.36±0.94**	**17.27±0.50**	**0.82±0.04**
150	41.68	41.25±1.04	17.14±0.33	0.43±0.02
180	37.90	37.66±0.96	16.23±0.73	0.24±0.01
210	35.55	35.32±0.53	15.55±0.40	0.23±0.01

Pulping conditions:

Time from ambient temperature to 105 ^{0}C = 45 min
Time from 105 ^{0}C to 135 ^{0}C = 35 min
Time at 130 ^{0}C = Varied (60-210 min)
Bath ratio = 1:5
Temperature = 130 °C
Alkali dose = 12% (as Na_2O)

Table-3.6: Effect of moisture content on pulp yield, kappa number and rejects

Solid to liquor ratio	Unscreened pulp yield (%)	Screened pulp yield (%)	Kappa no.	Rejects (%)
1:1	44.46	38.57±0.56	24.74±0.77	5.89±0.16
1:2	44.12	41.15±1.16	21.33±0.65	2.97±0.08
1:3	44.19	42.27±0.72	19.55±0.52	1.92±0.04
1:4	**44.46**	**43.58±0.60**	**17.38±0.45**	**0.88±0.03**
1:5	42.96	42.25±0.48	16.94±0.48	0.71±0.03

Pulping conditions:

Time from ambient temperature to 105 ^{0}C = 45 min
Time from 105 ^{0}C to 135 ^{0}C = 35 min
Time at 130 ^{0}C = 120 min
Bath ratio = Varied (1:1 to 1.5)
Temperature = 130 °C
Alkali dose = 12% (as Na_2O)

Table-3.7: Comparison of soda, ethanol-soda and bio-soda pulping of *E. binata*

Alkali dose (%)	Addition of ethanol (%)	Screened pulp yield (%)	Kappa no.	Rejects (%)
Ethanol-soda pulping				
12	20	43.02±0.83	17.52±0.45	0.82±0.03
12	25	44.50±0.86	16.68±0.39	0.79±0.03
12	**30**	**47.48±0.72**	**16.13±0.34**	**0.65±0.02**
12	35	45.66±0.62	15.84±0.25	0.53±0.01
Bio-soda pulping				
12	—	42.76±0.57	16.12±0.54	0.75±0.02
Soda pulping				
12	—	43.58±0.75	17.38±0.40	0.88±0.04

Table-3.8: Comparison of crystallinity index and viscosity of pulp after soda pulping, organosolve pulping and bio-pulping

Types of pulping	Kappa number	Yield* (%)	Crystallinity index (%)	Pulp viscosity (cps)	Reducing sugars (mg/ml of black liquor)
Soda	17.38±0.37	43.58±0.76	43.39±0.42	21.54±0.19	2.27±0.07
Bio-soda	16.12±0.40	42.76±0.68	42.30±0.54	23.16±0.21	1.94±0.05
Ethanol-soda	16.13±0.33	47.48±0.72	46.21±0.47	28.22±0.28	1.62±0.03

*Oven dry basis

Table-3.9: Comparison of brightness and strength properties after soda, ethanol-soda and bio-soda pulping of *E. binata*

Alkali dose (%)	Addition of ethanol (%)	Brightness (%) ISO	Tensile index (Nm/g)	Double fold (Numbers)	Burst index ($kPam^2/g$)	Tear index (mNm^2/g)
Ethanol-soda pulping						
12	20	41.3±0.3	68.56±2.14	293±4	6.53±0.17	12.41±.27
12	25	43.2±0.2	85.56±2.18	326±6	6.86±0.16	11.76±0.23
12	**30**	**43.9±0.4**	**89.24±2.25**	**344±7**	**8.49±0.15**	**11.76±0.19**
12	35	43.9±0.4	81.62±1.94	302±5	7.18±0.18	10.38±0.18
Bio-soda pulping						
12	—	41.4±0.3	84.35±1.96	288±4	7.15±0.13	11.38±0.20
Soda pulping						
12	—	37.3±0.5	67.51±2.07	194±3	6.27±0.14	13.06±0.22

Note: All the pulps were beaten at 35±1 °SR, +/- refers standard deviation, +/- shows percentage variations at optimum conditions

Figure-3.1: Showing the image of *E. binata*

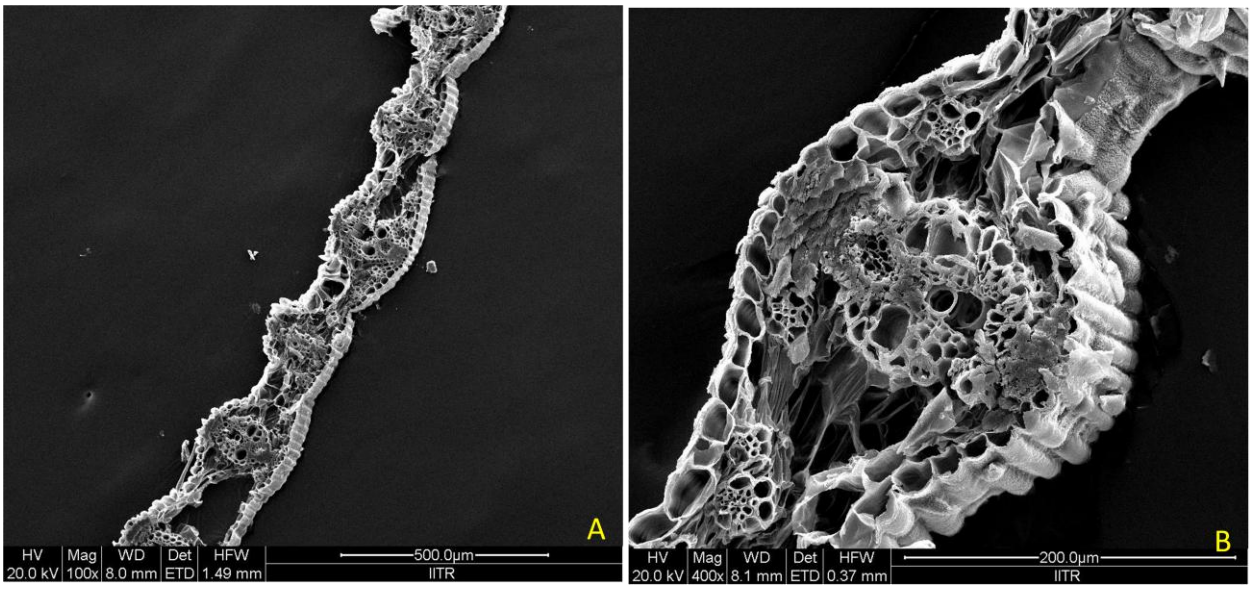

Figure-3.2: SEM image of transverse section of *E. binata*, (A) at 100 magnification, (B) at 500 magnification

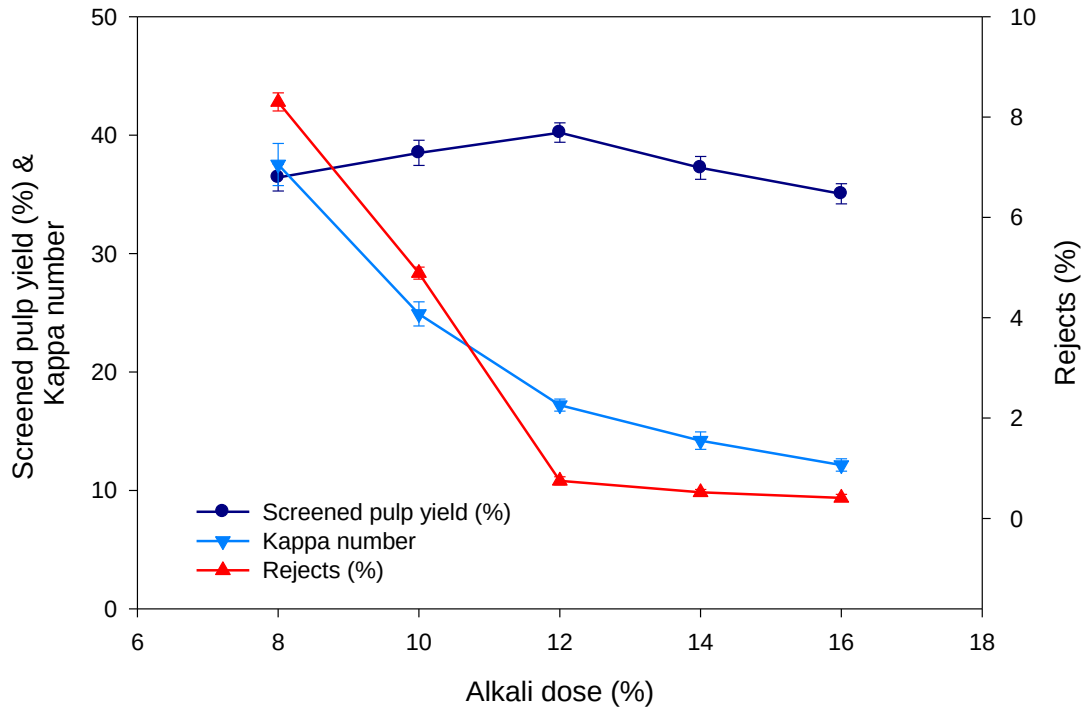

Figure-3.3: Effect of active alkali charge on screened pulp yield and kappa number

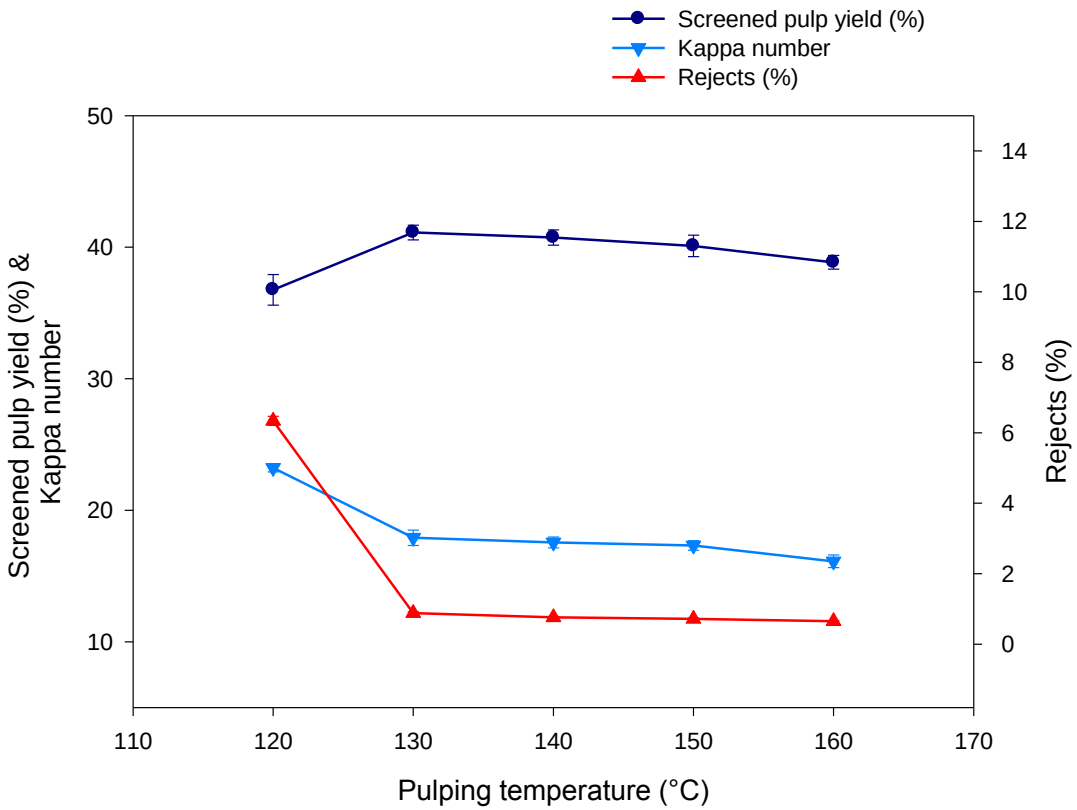

Figure-3.4: Effect of pulping temperature on screened pulp yield and kappa number

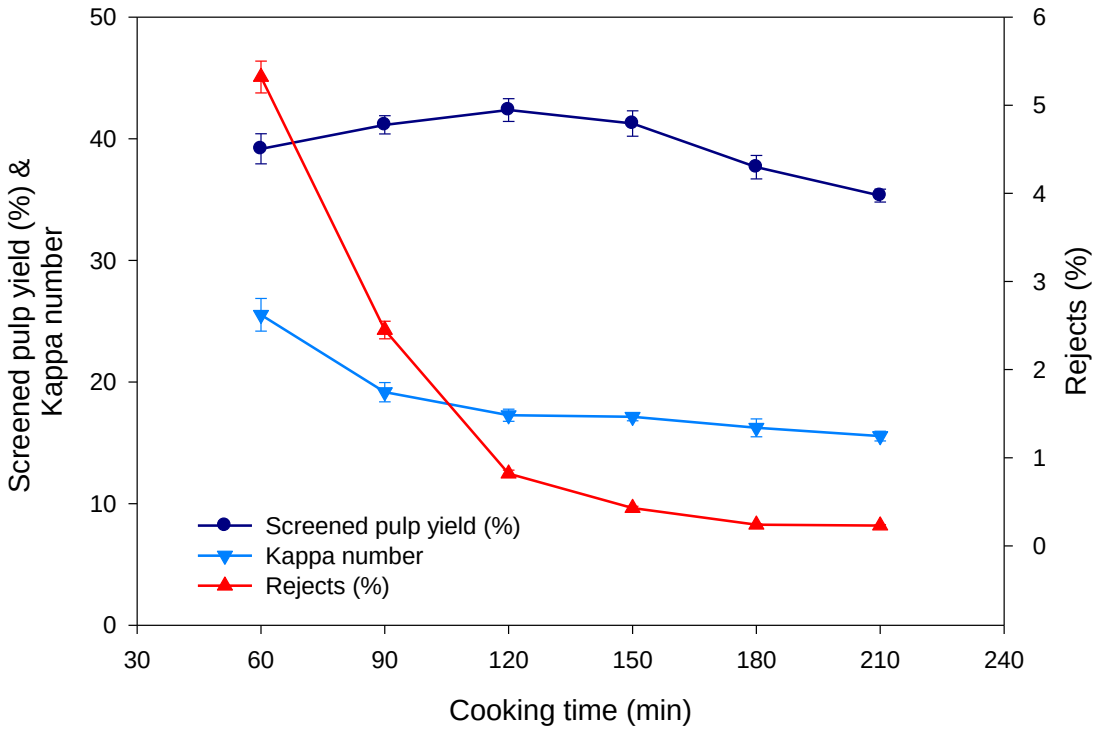

Figure3.5: Effect of cooking time on screened pulp yield and kappa number

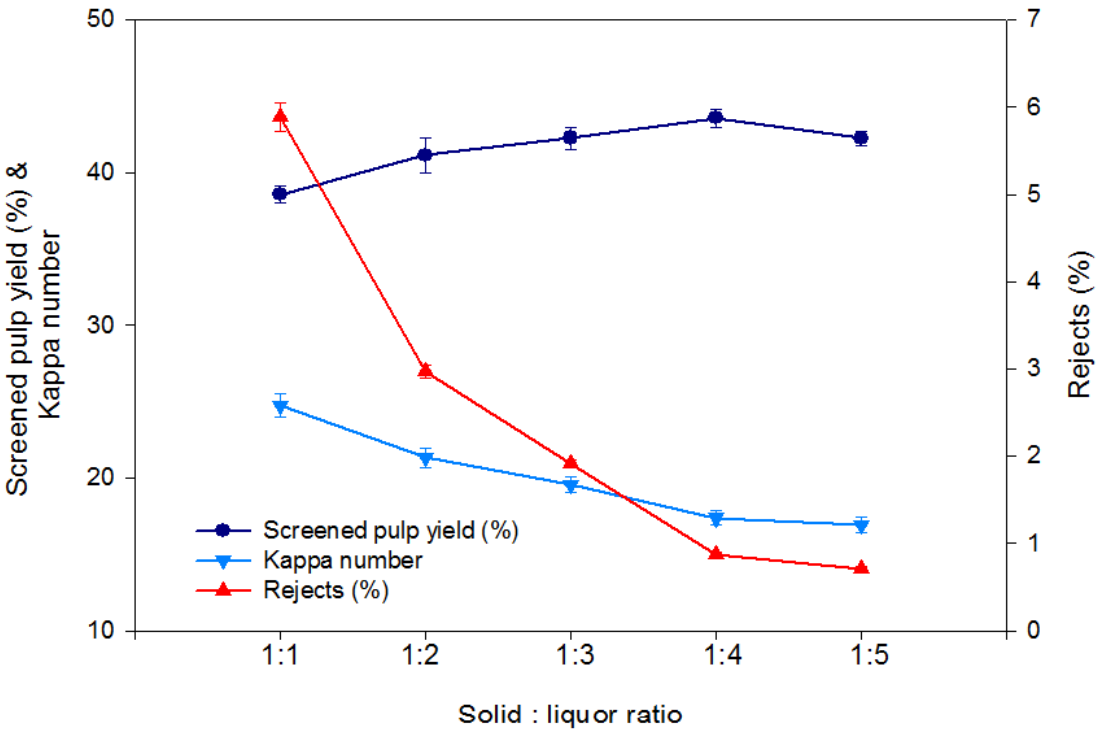

Figure-3.6: Effect of moisture ratio on screened pulp yield and kappa number

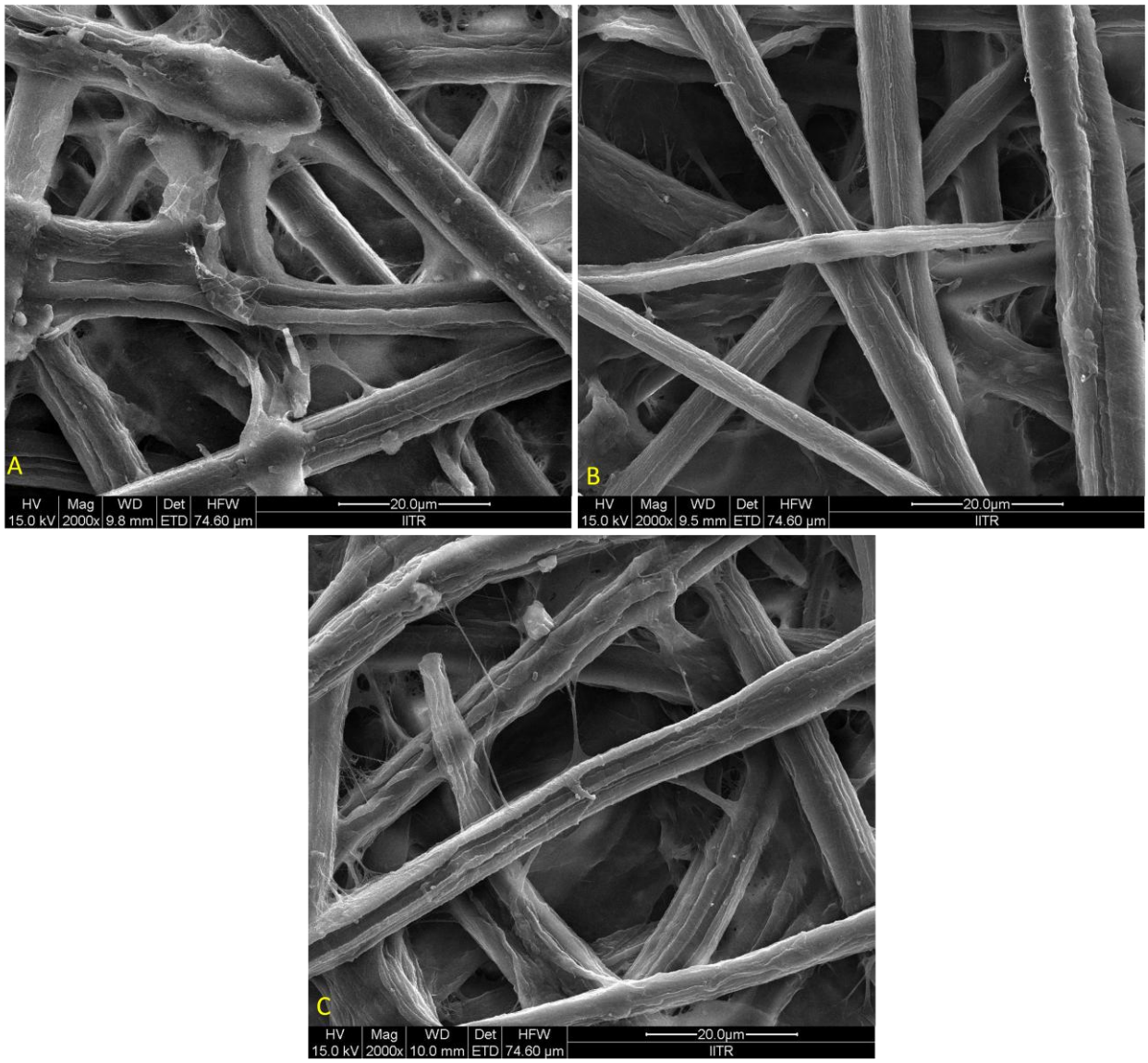

Figure-3.7: SEM image of (A) soda pulps and (B) bio-soda pulp (C) ethanol-soda pulp

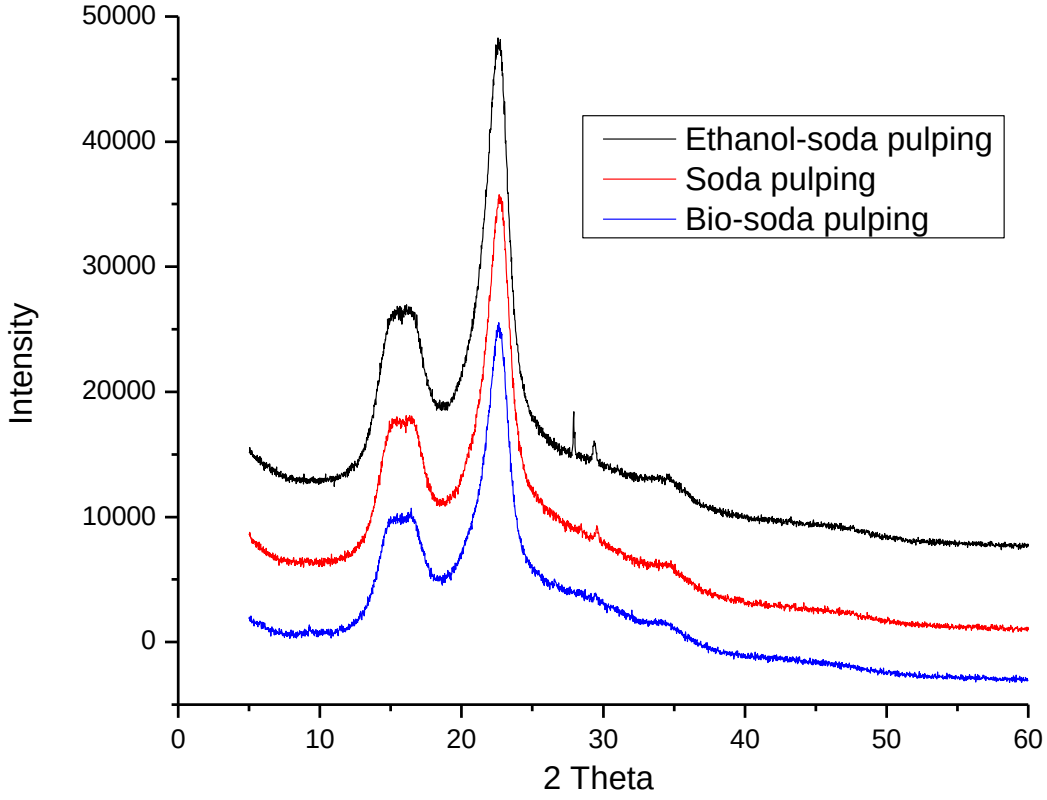

Figure-3.8: XRD analysis of soda, bio-soda and ethanol-soda pulps

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CHAPTER 4

BLEACHING STUDIES OF ETHANOL-SODA PULP OF *E. BINATA*

4.1: Introduction

The fibrous material obtained after pulping, consisting mainly of cellulose, is defined as pulp. Cellulose and hemicelluloses are inherently white and do not contribute towards colour. The colour of pulp is mainly contributed by chromophoric groups present in lignin. Although, most of lignin is removed during bulk delignification phase of chemical pulping processes and remainder lignin associated with secondary wall layers of the fibers (residual lignin) is removed during multi-stages bleaching sequences. The complete removal of residual lignin from pulp is carried out by using different oxidative agents for manufacturing of white varieties of paper [1, 2]. In general, the paper used for wrapping and packaging purposes and certain varieties of absorbent grades need not bleaching because optical properties are less important. The chemicals generally used for bleaching include oxidants like chlorine, chlorine dioxide, sodium hypochlorite, hydrogen peroxide, oxygen and ozone and alkali (sodium hydroxide) is used as in extraction stage of pulp. Sodium hydrosulfite (sodium dithionite), a reducing agent have been used in the brightening of mechanical pulps [1, 3, 4] i.e. conversion of chromophoric groups into leucochromophoric groups. The effectiveness of a reaction is measured in terms of pulp brightness, residual lignin content and residual chemical. To achieve sufficient removal of lignin by the use of any one of the chemicals in a single stage is not feasible. Therefore, bleach chemicals are used consecutively with intermediate washing steps in between the stages for primary objective of obtaining highest brightness. The secondary objectives are high brightness stability and high cellulose content and these objectives must be met without any significant loss in strength properties [3].

Elemental chlorine free (ECF) and total chlorine free (TCF) bleaching have brought about a major technological revolution in the pulp and paper industry which has led to the production of environmentally benign paper and mitigation of environmental emissions [1, 5]. Chlorine dioxide is the most widely used chemical to replace the chlorine in bleach plant. In ECF bleaching, Cl_2 is replaced with ClO_2 which reduces the formation and discharge of

chlorinated organic materials into the aquatic environment [6]. ClO_2 is 2.5 times more powerful oxidizer compared to Cl_2, and that it preserves cellulose and attacks on lignin more selectively. Therefore, pulp produced by ClO_2 bleaching is brighter and stronger than that produced by Cl_2 bleaching itself [7]. By substituting chlorine dioxide at levels of 70 to 100%, an apparent decrease of 80 to 90% in the level of chlorinated organic was found in mill effluent along with the reduction in dioxins to "non-detect" levels [7]. The first chlorine dioxide and alkaline extraction stages together account approximately 90% of lignin removal [6].

In TCF bleaching, oxygen, ozone and hydrogen peroxide appear to be advantageous from environmental point of view related to traditional chlorine free bleaching. Oxygen delignification stage is used after pulping and washing to remove additional lignin before bleaching. In oxygen delignification, molecular oxygen reacts with pulp in alkaline conditions and allows extended delignification of chemical pulp without a serious loss in pulp yield and with positive environmental impacts in presence of Epsom salt ($MgSO_4$), which is a carbohydrate stabilizer [6, 8, 9]. Oxygen delignification stage before bleaching reduces the amount of bleaching chemicals and the NaOH required during the first extraction stage. It also protects the pulp against degradation [10, 11], because an effort in greater reduction in kappa number is expected to lead to degradation of carbohydrates in the pulp and loss of pulp strength [12]. Oxygen delignification results higher brightness with equivalent amount of chemicals, lower rejects and lower water consumption and subsequent effluent discharge due to the greater recycling potential of oxygen stage effluents [13]. The drawback of oxygen delignification is that its effectiveness is limited to 50% of delignification. Beyond this level, severe degradation of cellulose occurs, causing deterioration of pulp viscosity and physical strength properties [3, 13-15].

Hydrogen peroxide (H_2O_2) is well known for bleaching of high lignin wood pulp and it reacts with lignin under alkaline conditions. Hydroperoxide anion (HOO^-) is the active species which is formed in alkaline conditions and is responsible for bleaching action of hydrogen peroxide. Hydroperoxide anion reacts with coloured ethylenic or carbonyl-containing structures in lignin [16, 17].

$$H_2O_2 + HO^- \leftrightarrow HOO^- + H_2O$$

Where $pK_a = 11.6$ at 25 °C

Hydrogen peroxide has a major role in ECF and TCF bleaching approaches of chemical and mechanical pulps [18-20], being used both as an alkaline bleaching stage and as reinforcement for alkaline extraction. H_2O_2 acts as a true bleaching agent. Compared with oxygen delignification, H_2O_2 delignification appears to provide better color reduction because of its specific action on chromophores [3]. Meeting the tough environmental demands of Best Available Technology (BAT), set up by the European commission seems to be quite possible through these modern bleaching sequences of ECF and TCF [21]. Hydrogen peroxide activity is adversely affected by presence of transition metals therefore, a chelating agent is required to use before P-stage [22].

Enzymatic bleaching can be a simple and economic alternative for cleaner pulp production [23]. The pretreatment of pulp with xylanase is known as "prebleaching" or "bleach boosting" since, it improves the penetration of bleaching chemicals by breaking the xylan network, which help in removing the trapped lignin from pulp fibre rather than removing lignin directly or attacking the lignin-based chromophores [23, 24]. Use of xylanase in bleaching is cost effective alternative for paper industry which offers various benefits in bleaching. Xylanase bleaching can significantly improves the final pulp brightness of bleached pulp along with reduction in bleaching cost, when it is used with ozone and hydrogen peroxide (total chlorine free bleaching) [23, 25]. Xylanase is very effective in decreasing the bleaching chemicals like chlorine or chlorine dioxide which can also lower the AOX in effluent discharge up to 25% [23, 26-28].

The present study aims at using crude enzyme preparations from *Aspergillus flavus* ARC-12 and *Schizophyllum commune* ARC-11 in pre-bleaching of ethanol-soda pulp of *E. binata* and to study its effect on conventional, ECF, and TCF bleaching sequences in terms of pulp brightness, viscosity, physical strength properties and pollution load.

4.2: Materials and methods

4.2.1: Optimization of various operating parameters for enzymatic pre-bleaching

Unbleached ethanol-soda pulp of *E. binata* was pre-bleached by xylanase and various operating parameters such as enzyme dose, consistency and reaction time were optimized. Pre-bleaching was performed with xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11 and

their impact on kappa number of pulp, viscosity [29] and releases of chromophores [30] as well as reducing sugars [31] in filtrate were analyzed.

4.2.1.1: Enzyme dose

The ethanol-soda pulp from *E. binata* was treated with different doses of xylanase from *A. flavus* ARC-12 and *S. commune* ARC-11 at enzyme dose ranging from 0 to 14 IU/g while keeping other variables constant like, consistency 8% and reaction time 120 min. For *A. flavus* ARC-12, reaction temperature and pH were 6.0 and 50 °C respectively while for *S. commune* ARC-11 reaction temperature and pH were taken 55 °C and 5.0 respectively. Xylanase treated pulp was extracted with 1.2% NaOH (as such in E-stage) as per conditions shown in Table 4.1.

4.2.1.2: Reaction time

In the similar way, the unbleached ethanol-soda pulp of *E. binata* was treated with xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11 at different reaction time varying from 30 to 180 min while keeping other parameters constant like, enzyme dose (as optimized in subsection 4.2.1.1) and consistency of 8%. For *A. flavus* ARC-12, reaction temperature and pH were 6.0 and 50 °C respectively while for *S. commune* ARC-11 reaction temperature and pH were taken 55 °C and 5.0 respectively.

4.2.1.3: Consistency

In an another set of experiment, the unbleached ethanol-soda pulp of *E. binata* was treated with xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11 at different pulp consistency ranging from 2 to 12% with a difference of two units while keeping other parameters constant. Optimum values of xylanase dose and reaction temperature (as optimized previously) were used during optimization of consistency. For *A. flavus* ARC-12, reaction temperature and pH were 6.0 and 50 °C respectively while for *S. commune* ARC-11 reaction temperature and pH were taken 55 °C and 5.0 respectively.

4.2.1.4: Enzymatic pre-bleaching of pulp in bulk

Unbleached ethanol-soda pulp of *E. binata* was pre-bleached in bulk with crude enzymes from *A. flavus* ARC-12 and *S. commune* ARC-11 under optimized conditions as described in Table 4.5 & 4.6. For *A. flavus* ARC-12, reaction temperature and pH were 50 °C and 6.0 respectively while for *S. commune* ARC-11 reaction temperature and pH were taken 55

°C and 5.0 respectively. In each case, controls were repeated in the similar manner using buffer in place of crude xylanase preparation. After enzymatic treatment, extraction of pulp samples was carried out with 1.2% NaOH (on as such basis). Pulp samples were filtered through a four-layered muslin cloth and washed with tap water. The pulp filtrates were analyzed for chromophores and reducing sugars released and pulp samples after XE-stage were analyzed for kappa number, pulp brightness and viscosity.

4.2.2: Application of xylanase in multistage bleaching process

4.2.2.1: Conventional bleaching of pulp

Unbleached ethanol-soda pulp of *E. binata* was bleached by CEHH, X_1ECEHH, and X_2ECEHH bleaching sequences. In conventional bleaching sequence 'X_1' represented for xylanase from *A. flavus* ARC-12 and X_2 represented xylanase from *S. commune* ARC-11. 'C' stood for chlorination, 'E' for alkaline extraction, 'H_1' for hypochlorite 1st stage, 'H_2' for hypochlorite 2nd stage. The xylanase pre-bleaching was carried out at optimized conditions (Table 4.5 & 4.6). The disintegrated pulp slurry was diluted with tap water to maintain a consistency of 3%. 50% of the total chlorine demand was charged in the form of molecular chlorine in 'C' stage and remaining 50% was charged in hypochlorite 1st and 2nd stages respectively i.e. 50% in 'H_1' and 50% in 'H_2' stage. The chlorination ('C' stage) was carried out at 3% consistency and pH 2.0 adjusted with dilute H_2SO_4 at ambient temperature. The plastic bottles were capped tightly to avoid leakage of molecular chlorine and contents were mixed well. After 30 min of chlorination, the pulp samples were filtered through a four-layered muslin cloth. The rest of the filtrate was preserved at 4 °C for further analysis. Whereas, hypochlorite 1st and 2nd stages were conducted at following bleaching conditions: consistency 10%, temperature 45 °C, pH 11.5 and reaction time 60 min. Extraction stage (E) was conducted with NaOH (as such) on o.d. pulp basis at consistency 10% and temperature 60±2 °C for 60 min. Residual chlorine in the filtrate after chlorination stage and hypochlorite 1st and 2nd stages was calculated as per method described for analysis of bleach liquor except the volume of spent bleach liquor was increased to 100 ml and titrated with 0.1N $Na_2S_2O_3$ solution [32]

4.2.2.2: Elemental chlorine free (ECF) bleaching

Unbleached ethanol-soda pulp of *E. binata* was bleached by DEDP, X_1DEDP, and X_2DEDP bleaching sequences where stands 'X_1' represented xylanase from *A. flavus* ARC-12

and X_2 represented xylanase from *S. commune* ARC-11, 'D_1' and 'D_2' stood for chlorine dioxide 1st and 2nd stages respectively, 'E' for alkaline extraction stage, 'P' for hydrogen peroxide stage. The xylanase pre-treatment stage was carried out under optimized conditions (Table 4.5 & 4.6). After E-stage, samples were treated with 2% chlorine dioxide in 'D_1' and 'D_2' stages (o.d. pulp basis) (1.34% in 'D_1' and 0.66% in 'D_2' stage) at a consistency of 10% at 70 °C for 180 min and pH 4.2. In E-stage NaOH (as such) was conducted at 10% consistency, 60 °C for 60 min and pH 11.7. In DEDP bleaching sequence, the final stage i.e. peroxide (P) stage was carried out at 10% consistency, temperature 90 °C, pH 10.3 and reaction time 60 min in polythene bag with 0.5% H_2O_2, 0.1% MgSO4 (as a carbohydrate stabilizer) and 0.5% EDTA (to mask the activities of d-block elements/transition metals). All the chemicals were added on o.d. pulp basis. The strength of H_2O_2 was determined by the method of Vogel's [33].

4.2.2.3: Total chlorine free (TCF) bleaching

Ethanol-soda pulp of *E. binata* were bleached using QOPP, X_1QOPP and X_2QOPP bleaching sequences where 'X_1' stood for xylanase from *A. flavus* ARC-12 and X_2 represented xylanase from *S. commune* ARC-11, 'Q' for chelating stage, 'O' for oxygen delignification and 'P' for hydrogen peroxide stage. Ethanol-soda pulp samples were treated with 0.2% DTPA at a pulp consistency of 3% and pH 4.5 for 30 min at ambient temperature [34]. After xylanase and DTPA treatments, the pulp was subjected to O_2 delignification in a WEVERK rotary digester. The pulp samples were mixed with 0.1% MgSO4, 0.2% EDTA, 2% NaOH and 10% consistency and placed in a vessel at following conditions, oxygen pressure 5.0 kg/cm^2, temperature 90 °C, reaction time 45 min and pH 12.0. In peroxide stage, 1.5% peroxide charge was given with 2% NaOH and 0.1% $MgSO_4$ at consistency 10%, temperature 90 °C for 60 min and pH 11.8. In final stage, 1.5% H_2O_2 was applied on o.d. pulp basis at conditions described in Table 5.7. All the chemicals were added on o.d. pulp basis.

4.2.3: Preparation of laboratory hand sheets and evaluation of paper properties

The ethanol-soda bleached pulp samples of *E. binata* were evaluated for bleaching losses, viscosity (TAPPI T 230 om-08) and copper number (TAPPI T 430 om-88) as per TAPPI Standard Test Methods. The pulp pads were prepared on Büchner funnel (TAPPI T 218 sp-11) and tested for brightness (TAPPI T 452 om-08). Laboratory handsheets of 60 g/m^2 were prepared (TAPPI T 205 sp-02) and conditioned at a temperature of 27±2 °C and a relative

humidity of 65±2%. These laboratory handsheets were tested for various physical strength properties such as tear index (TAPPI T 414 om-98), tensile index (TAPPI T 494 om-01), burst index (TAPPI T 403 om-97), double fold (TAPPI T 423 cm-98) [29].

4.2.4: Analysis of bleach effluent

The effluent generated after each stage of bleaching sequence was collected and mixed in equal amounts and were analyzed for COD (closed reflux titrimetric method using Thermoreactor CR2010) [35] colour (Test method No-204A) as per standard methods for the examination of water and waste water, American Public Health Association, 1985 [36] and AOX by column method [37] with AOX Analyzer Dextar ECS 1200.

4.2.5: Statistical analysis

All the experiments were carried out in triplicate and experimental results were represented as the mean ± standard deviation of three experimental values.

4.3: Results and discussion

4.3.1. Optimization of various operating parameters for enzymatic pre-bleaching

4.3.1.1: Effect of xylanase doses

Tables 4.1 & 4.2 revealed the prebleaching studies by xylanase doses varying from 0-14 IU/g, keeping other variables constant. Xylanases from two fungal strains *A. flavus* ARC-12 and *S. commune* ARC-11 were utilized for prebleaching studies. The curves could be approximated by two straight lines. Up to an enzyme dose of 10 IU/g, the curves with steeper slope pertained to rapid release of sugars whereas the part of curve with gentler slope pertained to the slow release of sugars. Both parts of the curves were having different velocity constants. The reducing sugars released increased with increasing xylanase dose during enzymatic pre-bleaching of pulp. Reducing sugars released at an enzyme dose of 10 IU/g were 4.57 and 2.52 mg/g for xylanase from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively beyond that, there were slight increments in reducing sugars (Figures 4.1 & 4.3). The released of chromophores were also measured during prebleaching at three different wavelengths i.e. 237 and 280 and 465 nm with spectrophotometer. The release of chromophores during prebleaching might be correlated with reduction in kappa number [30]. Xylanase hydrolyze the xylan and

soluble oligosaccharides during bleaching therefore, release of reducing sugars and chromophores were found to increase on increasing the xylanase doses [38].

Tables 4.1 & 4.2 showed that the absorbance at a wave length of 237 nm increased up to a xylanase dose of 10 IU/g from both the fungal strains, it was because of release of phenolic compounds or chromophores and beyond that increase in enzyme did not show any significant increase in absorbances (Figure 4.2 & 4.4). At a wave length of 465 nm, absorbance increased up to an enzyme dose of 10 IU/g, which was due to the release of hydrophobic compounds [39-41]. This confirms that xylanase acts on lignin carbohydrate complex (LCC) and degrades lignin-hemicellulose linkages [42], consequently degraded chromophores releases into the effluent [41, 43].

The extraction with 1.5% NaOH was performed after pretreatment of ethanol-soda pulp with xylanases from both the fungal strains and its effect on kappa number, brightness and viscosity were observed. The maximum decrease in kappa number of ethanol-soda pulp of *E. binata* was at an enzyme dosage of 10 IU/g of xylanase from both fungal strains *A. flavus* ARC-12 and *S. commune* ARC-11. The decrease in kappa number was 18.51 and 14.81% by xylanases (10 IU/g) from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively compared to control. Further increase in enzyme dose did not affect kappa number significantly. Similarly, pulp brightness improved by 4.2 and 2.9% (ISO) compared to control, at an enzyme dosage of 10IU/g from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively. Further, there were no major changes in brightness on increasing enzyme dose above 10 IU/g. Lignin-carbohydrate complexes were loosened and dissolved due to the action of xylanases. Alkali extraction was carried out to wash out the trapped LCC in the pulp [44, 45]. The maximum increase in pulp viscosity was 4.96 and 3.90% respectively with enzymes from *A. flavus* ARC-12 and *S. commune* ARC-11 at enzyme dose of 10 IU/g. Xylanase hydrolyzed xylan which is a polymer with low degree of polymerization and its removal resulted an improvement in average molecular weight of polymer system which increased the pulp viscosity [21, 46]. Ragauskas et al. [47] concluded that improvement in the pulp viscosity resulted by accumulation of high molecular weight polysaccharides, which occurred when xylan was eliminated selectively. Paice et al. [46] reported an improvement of 17.41% in pulp viscosity of hardwood kraft pulp after pretreatment by xylanase from *E. coli* at an enzyme dose of 5 U/ml. Garg et al. [48] studied prebleaching of wheat straw pulp by xylanase from *Bacillus stearothermophilus* SDX

and reported 7.14% reduction in kappa number, 4.75% improvement in brightness at 60 °C, pH 9.0 and reaction time of 120 min.

4.3.1.2: Effect of reaction time

Tables 4.3 & 4.4 showed the effect of reaction time on prebleaching of ethanol-soda pulp of *E. binata* by xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively. Reaction time varied from 0 to 180 min with a difference of 30 min while keeping other variables constant like an enzyme dose 10IU/g, temperature 50 °C, consistency 10% and pH 6.0 for *A. flavus* ARC-12 and an enzyme dose 10IU/g, temperature 55 °C, consistency 10% and pH 5.0 for *S. commune* ARC-11 during pre-bleaching of ethanol-soda pulp of *E. binata.* On increasing reaction time up to 120 min release of reducing sugars was increased and release of reducing sugars continued after 120 min but at a comparatively lower level (Figures 4.5 & 4.7). The later phenomenon may be due to hydrolysis of soluble xylooligosaccharides which were released due to initial depolymerization of the xylan [18, 41].

The majority of chromophores and colour were removed before a reaction time of 120 min for xylanases from both the fungal strains *A. flavus* ARC-12 and *S. commune* ARC-11 respectively and beyond that insignificant increase was observed (Figures 4.6 & 4.8). Thus, the released of chromophores in filtrate increased with decreasing kappa number of pulp [49]. Similar trends for release of chromophores in filtrate after xylanase pre-treatment were observed by various investigators [39, 41]. Beg et al. [39] reported maximum release of chromophores from eucalyptus kraft pulp at wavelengths 237 and 435 nm after 2 h of reaction time of xylanase from *Streptomyces* sp. QG-11-3.

The maximum reduction in kappa number of ethanol-soda pulp of *E. binata* was observed at reaction time of 120 min for xylanase from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively. Further, an increase in reaction time showed insignificant change in kappa number. The reduction in kappa number after reaction time 120 min was 15.82 and 17.30% by xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively. The maximum viscosity of ethanol-soda pulp of *E. binata* was 29.7 and 29.3 cps with xylanases from *Aspergillus flavus* ARC-12 and *S. commune* ARC-11 respectively at a reaction time of 120 min. The improvement in pulp brightness was 4.4 and 3.3% with xylanases *A. flavus* ARC-12 and *S. commune* ARC-11 respectively at 120 min of reaction time. Khandeparkar et al. [41]

studied the prebleaching of kraft pulp using xylanase from *Arthrobacter* sp. MTCC 5214 and reported 19.6% reduction in kappa number and 9.6% improvement in brightness after the reaction time of 2 h at an enzyme dose of 20 IU/g. Sanghi et al. [50] observed improvement in the brightness (3.3%) and reduction kappa number (4.62%) of kraft pulp by pretreatment with xylanase from *Bacillus subtilis* at an enzyme dose of 6 IU/g, 10% consistency, 55 °C temperature and reaction time of 2 h.

4.3.1.3: Effect of consistency

Tables 4.4 & 4.5 represented the effect of consistency on prebleaching of ethanol-soda pulp of *E. binata* by xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11. The effect of consistency varying from 0 to 12% with a difference of 2% while maintaining while keeping other variables constant like an enzyme dose 10IU/g, temperature 50 °C, reaction time 120 min and pH 6.0 for *A. flavus* ARC-12 and an enzyme dose 10IU/g, temperature 55 °C, reaction time 120 min and pH 5.0 for *S. commune* ARC-11 during pre-bleaching of ethanol-soda pulp of *E. binata*. During pre-bleaching, the release in reducing sugars increased with increase in pulp consistency up to 10% and beyond that a slow increase was noticed (Figures 4.9 & 4.11). The filtrates were analyzed for chromophores at wavelengths of 237, 280 and 465 nm and maximum leaching of chromophores was observed at a consistency of 10% (Figures 4.10 & 4.12). The maximum reduction in kappa number by 24.07 and 25.30% was observed for xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11 at a consistency of 10%. The improvement in viscosity of ethanol-soda pulp of *E. binata* was found 7.80 and 6.73 % using xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively at a consistency of 10% compared to control. Similarly, maximum improvement in pulp brightness was 4.8 and 4.4% (ISO) was achieved at a pulp consistency of 10% during prebleaching of ethanol-soda pulp of *E. binata* using xylanase from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively. Cellulosic fibers contains mobile and immobile layers of water and the thickness of mobile layer gradually declines on increasing the consistency of pulp while only the thin immobile layer remains around the cellulosic fiber. Therefore, at higher consistency, the diffusion path length of reactant to the fiber declines [51-53]. Higher pulp consistency also assists the enzyme to stabilize and allowing it to remain active under more severe conditions, e.g. higher temperature and pH [54]. Pre-bleaching was performed at 10% consistency in several studies

such as kraft pulp [55-57], for non-woods such as bagasse soda-AQ pulp [58, 59] and wheat straw soda-AQ pulp [60, 61].

After optimization of variables, a xylanase dose of 10IU/g, reaction time of 120 min, and pulp consistency of 10% were found most suitable for prebleaching of ethanol-soda pulp of *E. binata* using xylanase from *A. flavus* ARC-12 and *S. commune* ARC-11 respectively. For *A. flavus* ARC-12, xylanase pretreatment reduced the kappa number from 16.2 to 12.3, improved brightness from 43.9 to 48.7% (ISO) and viscosity from 28.2 to 30.4 cps of unbleached ethanol-soda pulp of *E. binata*. For *S. commune* ARC-11, xylanase pretreatment reduced the kappa number from 16.2 to 12.1, improved brightness from 43.9 to 48.3% (ISO) and viscosity from 28.2 to 30.1 cps of unbleached ethanol-soda pulp of *E. binata.*

4.3.2. Effect of xylanase pre-treatment on conventional bleaching

Table 4.7 showed the results of CEHH, X_1ECEHH, and X_2ECEHH bleaching sequences for ethanol-soda pulp of *E. binata*. The brightness and viscosity of ethanol-soda pulp bleached by CEHH bleaching sequence were 81.2% (ISO) and 8.4 cps respectively.

The brightness of ethanol-soda pulp of *E. binata* bleached by X_1ECEHH and X_2ECEHH bleaching sequences improved by 1.6 and 0.8% (ISO), viscosity by 4.76 and 3.57% compared to CEHH bleaching sequence. The improvement in brightness of ethanol-soda pulp of *E. binata* may be explained as enzymes improves the accessibility of bleaching chemicals by hydrolyzing the xylan chain which is responsible for close adherence of lignin to cellulose network and facilitates the easier removal of lignin during bleaching [23, 28, 62-64]. The degree of polymerization of xylan is lower compared cellulose, removal of xylan results an increase the ratio of high DP cellulose which increases the viscosity of pulp [46, 65]. Total chlorine demand was calculated by kappa number of pulp and xylanase pretreatment followed by alkali extraction declined the total chlorine demand by 23.5 and 24.5% for ethanol-soda pulp of *E. binata* during X_1ECEHH and X_2ECEHH. Bleached pulp yield of bleaching sequences CEHH, X_1ECEHH and X_2ECEHH were 43.34, 44.74 and 44.86% respectively.

Table 4.8 revealed mechanical strength properties of pulps of *E. binata* bleached by CEHH, X_1ECEHH and X_2ECEHH bleaching sequences at a fixed beating level of 35 °SR. Tear index improved by 14.21 and 12.95% during X_1ECEHH and X_2ECEHH bleaching sequences compared to CEHH. Similarly, the increase in burst index was insignificant and tensile index,

and double fold numbers were improved marginally during X_1ECEHH and X_2ECEHH bleaching sequences compared to CEHH bleaching sequence. The copper numbers in X_1ECEHH and X_2ECEHH bleached pulps declined by 21.73 and 13.04% respectively compared to CEHH. The copper number is regarded as an index of impurities in paper, such as oxycellulose, hydrocellulose, lignin, and sugars, which have reducing properties. So, it is a useful criterion for the determination of changes associated with deterioration of pulp. Therefore, it may be considered as a factor having an indirect effect on the durability of the paper and it denotes the degree of damage to cellulose in paper [66, 67]. Xylanase pre-treatment mitigated the AOX by 21.49 and 28.50% during X_1ECEHH and X_2ECEHH bleaching sequences compared to CEHH bleaching sequence. Pre-bleaching with crude xylanase preparation made the entire process economical in terms of reduced chemical/chlorine demand and reduced environmental load, which is being generated by the production of aromatic organic halides. Sharma et al. [68] reported 34% reduction in AOX generation in effluent obtained during prebleaching with xylanase and laccase. Senior and Hamilton [69] studied the prebleaching of hardwood pulp with xylanase and observed 35-40 and 24% reduction in chlorination charge and AOX in E-stage respectively while the BOD/COD ratio was increased under the same bleaching conditions. In some other studies, prebleaching of pulp with xylanases from different microbial sources saved chlorine by 20% as reported by Dhillon and Khanna [70], 28% Li et al. [60], and 18.7% Saleem et al. [64].

Combined bleach effluent was also analyzed for COD load and colour which were found to increase in effluents generated during X_1ECEHH and X_2ECEHH bleaching sequences compared to CEHH. COD load was increased by 16.56 and 22.89% during X_1ECEHH and X_2ECEHH bleaching sequences respectively compared to CEHH. The colour in combined bleach effluent was increased by 10.02 and 15.39% during X_1ECEHH and X_2ECEHH bleaching sequences compared to CEHH bleaching sequence. The COD and colour in bleach effluent increased due to increased concentration of residual lignin carbohydrate complexes (RLCC) by the hydrolytic action of enzyme. Several researchers made the similar observations [65, 71]. The high proportion of degraded xylan was confirmed in the effluent by Onysko [72], Senior and Hamilton [69]. When *E. globulus* pulp was pretreated with xylanase in a bleaching sequence OD_1PD_2, the bleaching effluent colour increased by 27.76% [65].

4.3.3. Effect of xylanase pre-treatment on ECF bleaching

Table 4.9 showed the results and bleaching conditions of DEDP, X_1DEDP, and X_2DEDP sequences of ethanol-soda pulp of *E. binata* and its effect on brightness, viscosity and bleached pulp yield. The brightness of ethanol-soda pulp by DEDP, X_1DEDP, and X_2DEDP bleaching sequences were 82.6, 85.8 and 84.5% and pulp viscosity were 8.8, 9.1 and 9.0% cps respectively. Bleached pulp yield was improved up to 45.24 and 45.57% in bleaching sequences X_1DEDP, and X_2DEDP compared to DEDP bleaching sequence (44.30%).

Brightness and viscosity of DEDP ethanol-soda pulp improved by 1.4% (ISO) and 4.76% respectively compared to CEHH bleaching sequence. An overall improvement in mechanical strength properties was also noticed after DEDP bleaching sequences compared to CEHH bleaching sequences due to the less damaging action of chlorine dioxide towards cellulose than chlorine [73]. The combined effluent generated during DEDP bleaching sequence of ethanol-soda pulp of *E. binata* showed a decrease in COD by 5.42%, colour by 27.77%, and AOX by 80.37% respectively compared to CEHH bleaching sequence. Chlorine dioxide is a stronger bleaching agent than chlorine. During bleaching, the reactions with chlorine dioxide are highly oxidative, which reduce the amount of AOX generated. The atomic chlorine content of chlorine dioxide is lower than molecular chlorine, which also decreases AOX in effluent [21].

The brightness of X_1DEDP, and X_2DEDP bleached ethanol-soda pulp of *E. binata* increased by 3.2 and 1.9% (ISO) respectively compared DEDP bleaching sequences at the same chlorine dioxide charge. Xylanase treatment enhances the porosity of pulp fibres and which subsequently improves the accessibility of bleaching chemicals into the pulp compared untreated pulp [74]. It allows the lignin fragments to remove from the pulp. Therefore, higher brightness of pulp can be obtained by xylanase treatment at the same bleaching dosage [75, 76]. Pre-treatment of bagasse pulp with *T. lanuginosus* SSBP xylanase (DED bleaching sequence) increased the pulp brightness by 4.5% compared to control [77]. Pretreatment with a commercial enzyme (Xylanase-P) increased the brightness of bagasse pulp by 3.1% and softwood kraft pulp by 5.1% compared to control after ECF bleaching [59].

The viscosity of X_1DEDP, and X_2DEDP bleached *E. binata* ethanol-soda pulp increased by 3.40 and 2.27% respectively compared to DEDP bleaching sequence. It is well

established that the crude xylanase hydrolyzes xylan only and not cellulose chains in pulp [22, 65, 78]. Copper number decreased by 28.0 and 25.33% for *E. binata* ethanol-soda pulp after X_1DEDP, and X_2DEDP bleaching sequences compared to DEDP. Xylanase pre-treatment reduced the degree of damage to cellulose of the ethanol-soda pulp after full bleaching sequences in terms of reduction in copper number.

Table 4.10 showed a comparison of mechanical strength properties for bleached by DEDP, X_1DEDP, and X_2DEDP bleaching sequences. Tear index improved by 20.29 and 15.53% during X_1DEDP and X_2DEDP sequences compared to DEDP. While improvement in other mechanical strength properties like burst index and double fold numbers were insignificant except slight improvement in tensile index during bleaching sequences X_1DEDP and X_2DEDP compared to DEDP. The consumed ClO_2 during X_1DEDP and X_2DEDP bleaching sequences were mitigated by 2.98 and 3.82% respectively. Xylanase pretreatment reduced AOX generation by 23.80 and 19.04% after X_1DEDP, and X_2DEDP bleaching sequences respectively compared to DEDP. 4-O-methylglucuronic acid side chain of hemicelluloses is converted in to hexenuronic acid (HexA) during pulp cooking. Some authors indicated that the formation of AOX during bleaching has close relationship with HexA content of pulp [28, 79, 80]. Various studies proposed that HexA will consume the chlorine dioxide during bleaching. It is primarily the in-situ generated hypochlorous acid that reacts with HexA to form AOX [81, 82]. Furthermore, when hemicelluloses and HexA were removed from the fibre due to xylanase action, the lignin could easily react with ClO_2 and AOX generation decreased at the same dose of ClO_2 [28]. Nie [28] studied the xylanase-aided chlorine dioxide bleaching of bagasse pulp and concluded that lignin and HexA were the main sources of AOX generation and xylanase pretreatment removed HexA, mitigated AOX formation by 21.4–26.6% to achieve same level of brightness.

On contrary to this, the COD showed an increase by 9.87 and 7.96% respectively in combined bleached effluent obtained from X_1DEDP, and X_2DEDP bleached pulps compared to DEDP. The increase in COD of combined bleach effluent of xylanase prebleaching sequences may be explained due to dissolution of xylan and lignin fragments with carbohydrates compared to control [83]. Pretreatment of pulp with xylanase and its subsequent bleaching with sequence $CDED_1D_2$ improved various physical properties of the pulp i.e. viscosity, tensile strength, breaking length, burst factor, and tear factor and by 44%, 32%, 21%, 6%, and, 7%

respectively, which greatly improves the quality of the paper [55]. Lin et al. [74] studied prebleaching of wheat straw soda pulp with xylanase from *Bacillus halodurans* C-125 and reported reduction of ClO_2 by 10% in ECF bleaching sequence while maintaining the brightness and physical strength properties such as tear index, burst index and tensile index at same level.

4.3.4. Effect of xylanase pre-treatment on TCF bleaching

Table 4.11 depicted the results and bleaching conditions of QOPP, X_1QOPP and X_2QOPP bleaching sequences of ethanol-soda pulp of *E. binata*. The brightness of QOPP, X_1QOPP and X_2QOPP bleached ethanol-soda pulp were 82.1, 84.6, and 83.5% respectively and their respective viscosity values were 9.0, 9.3, and 9.5 cps. The pulp viscosity of QOPP bleached pulp was higher compared to CEHH and DEDP bleached pulps because of the fact that no chlorine containing chemical was used during QOPP bleaching sequence.

Brightness of *E. binata* ethanol-soda pulp increased by 2.5 and 1.4% (ISO) after X_1QOPP and X_2QOPP bleaching sequences respectively compared to QOPP bleaching sequence. Xylanase treatment improves the accessibility of bleaching chemicals into the pulp, which in turn enhances the brightness of the pulp after subsequent oxygen and hydrogen peroxide bleaching stages [74, 84]. The pulp viscosity of *E. binata* ethanol-soda pulp improved by 3.33 and 5.55% after X_1QOPP and X_2QOPP bleaching sequences compared to QOPP. The increase in pulp viscosity is due to removal of low molecular weight xylan by the action of xylanase [46, 78]. Bleached pulp yields were 42.94 and 42.65% after X_1QOPP and X_2QOPP bleaching sequences of ethanol-soda pulp of *E. binata* compared to QOPP (43.52%) bleaching sequence.

Table 4.12 showed comparisons of mechanical strength properties and combined effluent characteristics of QOPP, X_1QOPP and X_2QOPP bleaching sequences. Copper number of ethanol-soda pulp of *E. binata* decreased by 22.22 and 33.33% in X_1QOPP and X_2QOPP bleached pulps respectively compared to QOPP bleached pulp. Tear index increased by 3.67 and 3.25% after X_1QOPP and X_2QOPP bleaching sequences compared to QOPP bleaching sequence. Conversely, burst index remained almost constant. In the similar way, tensile index and double fold numbers were also decreased slightly after X_1QOPP and X_2QOPP bleaching sequences compared to QOPP bleaching sequence. Xylanase pretreatment of ethanol-soda pulp

of *E. binata* during X_1QOPP and X_2QOPP bleaching sequences increased the COD values by 15.87 and 9.42% respectively compared to QOPP bleaching sequence. The colour of combined bleach effluent generated during X_1QOPP and X_2QOPP bleaching sequences increased by 10.90 and 12.08% respectively compared to control. The increase in COD and colour of bleach effluent is due to the xylanase pre-treatment, since the hydrolytic action of xylanase leads to weakening of the carbohydrate bonds in the pulp and dissolution of lignin and hydrolyzed xylan into the media [65, 71].

Table-4.1: Optimization of xylanase dose (*A. flavus* ARC-12) for prebleaching of *E. binata* ethanol-soda pulp

Xylanase dose, IU/g	* Kappa number	Reducing sugars released, mg/g	*Viscosity, cps	Brightness, % (ISO)	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
0	16.2±0.42	–	28.2±0.11	43.9±0.3	–	–	–
4	15.1±0.30	1.10±0.04	28.5±0.14	45.2±0.4	0.321±0.012	0.285±0.008	0.210±0.004
6	14.8±0.28	1.98±0.05	28.9±0.15	46.3±0.2	0.381±0.011	0.314±0.009	0.288±0.009
8	14.3±0.23	3.04±0.09	29.4±0.10	47.3±0.5	0.432±0.017	0.358±0.016	0.322±0.010
10	**13.2±0.25**	**4.57±0.08**	**29.6±0.13**	**48.1±0.4**	**0.485±0.016**	**0.380±0.013**	**0.338±0.011**
12	13.5±0.27	5.81±0.17	29.2±0.15	48.3±0.3	0.490±0.019	0.421±.014	0.354±0.009
14	13.3±0.19	6.24±0.25	28.5±0.16	48.5±0.3	0.502±0.015	0.434±0.016	0.360±0.012

± Refers to standard deviation
Operational conditions: Extraction stage =1.2% NaOH at 70^{0}C temperature for 90 min, X-stage= enzyme dose varied, reaction time 120 min, pH 6.0, temperature 50±2 ^{0}C , consistency 8%

Table-4.2: Optimization of xylanase dose (*S. commune* ARC-11) for prebleaching of *E. binata* ethanol-soda pulp

Xylanase dose, IU/g	* Kappa number	Reducing sugars released, mg/g	*Viscosity, cps	Brightness, % (ISO)	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
0	16.2±0.40	–	28.2±0.14	43.9±0.5	–	–	–
4	15.2±0.32	0.52±0.04	28.4±0.15	44.4±0.4	0.243±0.014	0.202±0.006	0.141±0.009
6	14.8±0.31	0.95±0.05	28.7±0.10	44.9±0.3	0.207±0.015	0.243±0.008	0.167±0.010
8	14.3±0.21	1. 58±0.08	29.2±0.16	45.6±0.4	0.372±0.018	0.283±0.015	0.198±0.008
10	**13.8±0.20**	**2.52±0.10**	29.3**±0.13**	**46.8±0.5**	**0.414±0.019**	**0.332±0.016**	**0.225±0.010**
12	13.1±0.25	3.02±0.16	28.9±0.14	47.1±0.4	0.422±0.015	0.341±0.009	0.230±0.012
14	13.1±0.23	3.56±0.23	28.4±0.12	47.6±0.3	0.432±0.016	0.346±0.010	0.236±0.015

± Refers to standard deviation
Operational conditions: Extraction stage =1.2% NaOH at 70^{0}C temperature for 90 min, X stage= enzyme dose varied, reaction time 120 min, pH 5.0, temperature 55±2 ^{0}C, consistency 8%

Table-4.3: Optimization of reaction time (*A. flavus* ARC-12) for prebleaching of pulp of *E. binata*

Reaction time, min.	* Kappa number	Reducing sugars released, mg/g	*Viscosity, cps	Brightness, %(ISO)	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
30	15.8±0.17	3.23±0.11	28.6±0.10	45.3±0.4	0.324± 0.004	0.220± 0.003	0.106± 0.004
60	15.1±0.12	3.80±0.14	28.8±0.14	46.8±0.4	0.421± 0.003	0.302± 0.002	0.179± 0.002
90	14.4±0.13	4.62±0.18	29.2±0.11	48.0±0.5	0.463± 0.002	0.346± 0.003	0.262± 0.005
120	**13.3±0.09**	**5.37±0.22**	**29.7±0.10**	**48.3±0.4**	**0.489± 0.004**	**0.386± 0.004**	**0.328± 0.009**
150	13.1±0.08	5.78±0.24	29.5±0.12	48.6±0.5	0.494± 0.005	0.398± 0.008	0.345± 0.007
180	12.8±0.08	6.14±0.17	29.4±0.14	48.9±0.3	0.507± 0.006	0.411± 0.009	0.346± 0.010

± Refers to standard deviation
Operational conditions: Extraction stage =1.2% NaOH at 70^{0}C temperature for 90 min, X stage= enzyme dose 10 IU/g o.d. pulp , reaction time varied, pH 6.0, temperature 50±2 ^{0}C, consistency 8%

Table-4.4: Optimization of reaction time (*S. commune* ARC-11) for prebleaching of pulp of *E. binata*

Reaction time, min.	* Kappa number	Reducing sugars released, mg/g	*Viscosity, cps	Brightness, % (ISO)	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
30	15.6±0.19	3.14±0.14	28.5±0.12	44.8±0.3	0.203± 0.005	0.164±0 .005	0.102±0 .007
60	14.9±0.16	3.53±0.11	28.8±0.09	45.1±0.5	0.276± 0.006	0.231±0 .004	0.143±0 .005
90	14.5±0.11	4.21±0.16	28.9±0.11	46.6±0.4	0.352± 0.005	0.311±0 .004	0.185±0 .004
120	**12.9±0.08**	**4.83±0.24**	**29.3±0.12**	**47.2±0.5**	**0.425± 0.002**	**0.340±0 .003**	**0.228±0 .005**
150	13.1±0.09	5.91±0.23	29.2±0.14	47.4±0.3	0.434± 0.006	0.348±0 .007	0.231±0 .010
180	12.9±0.10	6.56±0.15	29.0±0.09	47.7±0.3	0.439± 0.008	0.354±0 .008	0.239±0 .012

± Refers to standard deviation
Operational conditions: Extraction stage =1.2% NaOH at 70^{0}C temperature for 90 min, X stage= enzyme dose 10 IU/g o.d. pulp , reaction time varied, pH 5.0, temperature 55±2 ^{0}C, consistency 8%

Table-4.5: Optimization of consistency for xylanase from *A. flavus* ARC-12 for prebleaching of pulp of *E. binata*

Cy %	*Kappa number	Reducing sugars released, mg/g	*Viscosity, cps	Brightness, % (ISO)	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
2	15.4±0.09	1.29±0.01	28.7±0.05	44.9±0.18	0.325± 0.004	0.205± 0.003	0.123± 0.004
4	15.3±0.07	3.26±0.09	28.9±0.11	45.3±0.23	0.372± 0.004	0.249± 0.004	0.168± 0.003
6	14.5±0.14	3.96±0.12	29.3±0.09	46.9±0.20	0.417± 0.002	0.297± 0.005	0.212± 0.006
8	13.2±0.12	4.70±0.18	29.7±0.08	47.5±0.35	0.458± 0.005	0.346± 0.008	0.260± 0.004
10	**12.3±0.08**	**5.28±0.20**	**30.4±0.10**	**48.7±0.27**	**0.517± 0.006**	**0.392± 0.009**	**0.275± 0.007**
12	11.8±0.10	5.83±0.15	30.2±0.06	48.6±0.34	0.530± 0.004	0.401± 0.012	0.279± 0.011

± Refers to standard deviation

Operational conditions: Extraction stage =1.2% NaOH at 70^{0}C temperature for 90 min, X stage= enzyme dose 10 IU/g o.d. pulp , reaction time 120 min, pH 6.0, temperature 50±2 ^{0}C, consistency varied 2-12%

Table-4.6: Optimization of consistency for xylanase from *S. commune* ARC-11 for prebleaching of pulp of *E. binata*

Cy %	*Kappa number	Reducing sugars released, mg/g	*Viscosity, cps	Brightness, % (ISO)	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
2	15.3±0.10	2.37±0.06	28.6±0.08	45.1±0.2	0.306± 0.005	0.236± 0.005	0.115± 0.005
4	15.9±0.08	3.22±0.11	29.1±0.12	45.6±0.3	0.353± 0.006	0.270± 0.003	0.141± 0.004
6	14.8±0.09	3.92±0.10	29.5±0.10	46.5±0.3	0.390± 0.007	0.314± 0.007	0.185± 0.008
8	13.3±0.10	4.83±0.15	29.7±0.09	47.3±0.4	0.426± 0.008	0.339± 0.005	0.222± 0.007
10	**12.1±0.12**	**5.64±0.19**	**30.1±0.11**	**48.3±0.3**	**0.464± 0.004**	**0.384± 0.004**	**0.273± 0.006**
12	11.7±0.10	6.05±0.18	29.9±0.08	48.5±0.5	0.477± 0.003	0.389± 0.010	0.285± 0.010

± Refers to standard deviation

Operational conditions: Extraction stage =1.2% NaOH at 70 $^\circ$C temperature for 90 min, X stage= enzyme dose 10 IU/g o.d. pulp , reaction time 120 min, pH 5.0, temperature 55±2 ^{0}C, consistency varied, 2-12%

Table-4.7: Effect of conventional bleaching on, brightness and viscosity of pulp of *E. binata*

Particulars	Bleaching sequence		
	CEHH	**X_1ECEHH**	**X_2ECEHH**
Unbleached pulp kappa number	16.1±0.3	16.1±0.3	16.1±0.3
Unbleached pulp brightness, % (ISO)	43.9±0.2	43.9±0.2	43.9±0.2
Unbleached pulp viscosity, cps	28.2±0.14	28.2±0.14	28.2±0.14
Xylanase stage (X)			
Amount of xylanase added (on o.d. pulp basis), IU/g	–	10	10
pH	–	6.0	5.0
Alkali extraction stage (E)			
NaOH applied, % (o.d. pulp basis)	–	1.2	1.2
Initial pH	–	11.2	11.2
Final pH	–	10.6	10.5
kappa number of xylanase treated pulp	–	12.3	12.1
Chlorination stage (C)			
Cl_2 applied, % (o.d. pulp basis)	2.0	1.53	1.51
Cl_2 consumed, % (o.d. pulp basis)	1.9	1.51	1.49
Amount of Cl_2 consumed, %	95	98.6	97.3
Final pH	1.7	1.8	2.1
Alkali extraction stage (E)			
NaOH applied, % (o.d. pulp basis)	0.76	0.581	0.581
Initial pH	11.2	11.6	11.5
Final pH	11.5	11.4	11.4
Hypochlorite stage (H_1)			
Hypo applied as available Cl_2, % (o.d. pulp basis)	1.0	0.76	0.75
Hypo consumed as available Cl_2, % (o.d. pulp basis)	0.94	0.72	0.70
Hypo consumed, %	96.0	96.0	94.6
Final pH	11.3	11.2	11.3
Hypochlorite stage (H_2)			
Hypo applied as available Cl_2, % (o.d. pulp basis)	1.0	0.76	0.75
Hypo consumed as available Cl_2, % (o.d. pulp basis)	0.94	0.72	0.70
Hypo consumed, %	94.0	94.7	93.3
Final pH	11.4	11.2	11.3
Total Cl_2 applied, % (o.d. pulp basis)	4.0	3.07	3.02
Total Cl_2 consumed, % (o.d. pulp basis)	3.8	2.96	2.90
Total Cl_2 consumed on Cl_2 basis, %	95.0	96.41	96.02
Bleached pulp yield, %	43.34±1.3	44.78±1.4	44.86±1.5
Pulp brightness, % (ISO)	81.2±0.4	82.8±0.5	82.0±0.3
Pulp viscosity, cps	8.4±0.013	8.9±0.010	8.7±0.012

Bleaching conditions	X_1	X_2	C	E	H
Consistency, %	10	10	3	10	10
Temperature, ^{0}C	50±2	55±2	Ambient	60±2	45
Time, min	120	120	30	60	60

± refers standard deviation

Table-4.8: Comparison of mechanical strength properties and combined effluent characteristics generated during conventional bleaching of pulp of *E. binata*

Sl. No	Particulars	CEHH	X_1ECEHH	X_2ECEHH
1	Pulp brightness, (ISO), %	81.2±0.4	82.8±0.3	82.0±0.3
2	Pulp viscosity, cps	8.4±0.12	8.9±0.10	8.7±0.09
3	Copper number	0.23±0.003	0.18±0.004	0.20±0.004
4	Beating level, ^{0}SR	35±1	35±1	35±1
5	Tear index, mNm^2/g	10.34±0.19	11.81±0.17	11.68±0.16
6	Burst index, kPam^2/g	7.62±0.23	7.98±0.18	7.88±0.19
7	Tensile index, Nm/g	80.34±2.1	83.38±1.6	83.14±1.8
8	Double fold, number	299±6	310±7	308±6
9	COD, mg/L	1328±26	1548±23	1632±32
10	Color, PTU	2124±32	2337±35	2451±29
11	AOX, kg/t	2.14±0.02	1.68±0.01	1.53±0.01

Table-4.9: Effect of xylanase pretreatment on, brightness and viscosity of pulp of *E. binata*

Particulars				4.2.1.5: Bleaching sequence		
				DEDP	**X_1DEDP**	**X_2DEDP**
Unbleached pulp kappa number				16.1±0.3	16.1±0.3	16.1±0.3
Unbleached pulp brightness, % (ISO)				43.9±0.2	43.9±0.2	43.9±0.2
Unbleached pulp viscosity, cps				28.2±0.14	28.2±0.14	28.2±0.14
Xylanase stage (X)						
Amount of xylanase added (on o.d. pulp basis), IU/g				–	10	10
pH				–	6.0	5.0
Chlorine dioxide stage (D_1)						
ClO_2 applied as available Cl_2, % o.d. pulp basis)				1.34	1.34	1.34
ClO_2 consumed as available Cl_2, % (o.d. pulp basis)				1.26	1.22	1.21
ClO_2 consumed on Cl_2 basis, %				94.02	91.04	90.2
Final pH				4.1	4.0	4.0
Alkali extraction stage (E)						
NaOH applied, % (o.d. pulp basis)				1.2	1.2	1.2
Initial pH				11.1	10.8	10.7
Final pH				11.3	11.2	11.2
Chlorine dioxide stage (D_2)						
ClO_2 applied as available Cl_2, % (o.d. pulp basis)				0.660	0.660	0.660
ClO_2 consumed as available Cl_2, % (o.d. pulp basis)				0.602	0.598	0.596
ClO_2 consumed, %				91.21	90.60	90.3
Final pH				4.1	4.1	4.1
Peroxide stage (P)						
H_2O_2 applied, % (o.d. pulp basis)				0.5	0.5	0.5
EDTA applied, % (o.d. pulp basis)				0.5	0.5	0.5
$MgSO_4$ applied, % (o.d. pulp basis)				0.1	0.1	0.1
Final pH				10.4	10.9	10.8
Total ClO_2 applied, % (o.d. pulp basis)				2.0	2.0	2.0
Total ClO_2consumed, % (o.d. pulp basis)				1.862	1.818	1.806
Total ClO_2consumed on Cl_2 basis, %				93.1	90.9	90.3
Bleached pulp yield, %				44.30±1.4	45.24±1.5	45.57±1.1
Pulp brightness, % (ISO)				82.6±0.2	85.8±0.3	84.5±0.2
Pulp viscosity, cps				8.8±0.008	9.1±0.016	9.0±0.012
Bleaching conditions	X_1	X_2	D_1	E	D_2	P
Consistency, %	10	10	10	10	10	10
Temperature, ^{0}C	50±2	55±2	70±2	60±2	70±2	90±2
Time, min	120	120	180	60	180	60

± refers standard deviation

Table-4.10: Comparison of mechanical strength properties and combined effluent generated during ECF bleaching of *E. binata* pulp

Sl. No.	Particulars	DEDP	X_1DEDP	X_2DEDP
1	Pulp brightness, % (ISO)	82.6±0.4	85.8±0.2	84.5±0.4
2	Pulp viscosity, cps	8.8±0.15	9.1±0.11	9.0±0.17
3	Copper number	0.15±0.003	0.11±0.002	0.11±0.004
4	Beating level, ^{0}SR	35±1	35±1	35±1
5	Tear index, mNm^2/g	11.38±0.13	13.69±0.11	13.15±0.14
6	Burst index, kPam^2/g	7.76±0.16	7.93±0.15	7.85±0.17
7	Tensile index, Nm/g	81.77±1.73	82.78±1.46	82.43±1.87
8	Double fold, number	302±6	306±4	305±5
9	COD, mg/L	1256±22	1380±19	1356±25
10	Color, PTU	1534±34	1733±28	1629±37
11	AOX, kg/t	0.42±0.008	0.32±0.007	0.34±0.007

± refers standard deviation

Table-4.11: Effect of TCF bleaching on pulp shrinkage, brightness and viscosity of *E. binata* pulp

4.2.2.4: Particulars	Bleaching sequence		
	QOPP	**X_1QOPP**	**X_2QOPP**
Unbleached pulp kappa number	16.1±0.3	16.1±0.3	16.1±0.3
Unbleached pulp brightness, % (ISO)	43.9±0.2	43.9±0.2	43.9±0.2
Unbleached pulp viscosity, cps	28.2±0.14	28.2±0.14	28.2±0.14
Xylanase stage (X)			
Amount of xylanase added (on o.d. pulp basis), IU/g	–	10	10
pH	–	6.0	5.0
Chelating stage (Q)			
DTPA applied, % (o.d. pulp basis)	0.2	0.2	0.2
Final pH	4.6	4.6	4.6
Oxygen stage (O)			
O_2 pressure, kg/cm^2	5.0	5.0	5.0
$MgSO_4$ applied, % (o.d. pulp basis)	0.1	0.1	0.1
EDTA applied, % (o.d. pulp basis)	0.2	0.2	0.2
NaOH applied, % (o.d. pulp basis)	2.0	2.0	2.0
Final pH	12.0	12.0	12.0
Peroxide stage (P_1)			
H_2O_2 applied, % (o.d. pulp basis)	1.5	1.5	1.5
H_2O_2 consumed, % (o.d. pulp basis)	1.47	1.45	1.46
DTPA applied, % (o.d. pulp basis)	0.5	0.5	0.5
$MgSO_4$ applied, % (o.d. pulp basis)	0.1	0.1	0.1
Final pH	11.8	11.8	11.7
Peroxide stage (P_2)			
H_2O_2 applied, % (o.d. pulp basis)	1.5	1.5	1.5
H_2O_2 consumed, % (o.d. pulp basis)	1.43	1.26	1.30
DTPA applied, % (o.d. pulp basis)	0.5	0.5	0.5
$MgSO_4$ applied, % (o.d. pulp basis)	0.1	0.1	0.1
Final pH	11.8	11.8	11.7
Total H_2O_2 applied, % (o.d. pulp basis)	3.0	3.0	3.0
Total H_2O_2 consumed, % (o.d. pulp basis)	2.90	2.71	2.76
Bleached pulp yield, %	43.52±0.39	42.94 ±0.48	42.65±0.55
Pulp brightness ,% (ISO)	82.1±0.3	84.6±0.4	83.5±0.3
Pulp viscosity, cps	9.0±0.011	9.3±0.023	9.5±0.014

Bleaching conditions	X1	X2	Q	O	P
Consistency, %	10	10	3	10	10
Temperature, ^0C	50±2	55±2	Ambient	90±2	90±2
Time, min	120	120	30	45	60

± refers standard deviation

Table-4.12: Comparison of mechanical strength properties and combined effluent generated during TCF bleaching of *E. binata* pulp

Sl. No.	Particulars	QOPP	X_1QOPP	X_2QOPP
1	Pulp brightness, % (ISO)	82.1±0.4	84.6±0.3	83.5±0.3
2	Pulp viscosity, cps	9.0±0.11	9.3±0.17	9.5±0.12
3	Copper number	0.09±0.002	0.07±0.001	0.06±0.002
4	Beating level, ^{0}SR	35±1	35±1	35±1
5	Tear index, mNm^2/g	11.98±0.21	12.42±0.25	12.37±0.16
6	Burst index, kPam^2/g	7.70±0.19	7.45±0.17	7.38±0.15
7	Tensile index, Nm/g	82.94±1.72	81.15±2.40	80.34±1.84
8	Double fold, number	309±4	294±3	290±5
9	COD, mg/L	1348±28	1562±31	1475±27
10	Color, PTU	1862±30	2065±36	2087±31

± refers standard deviation

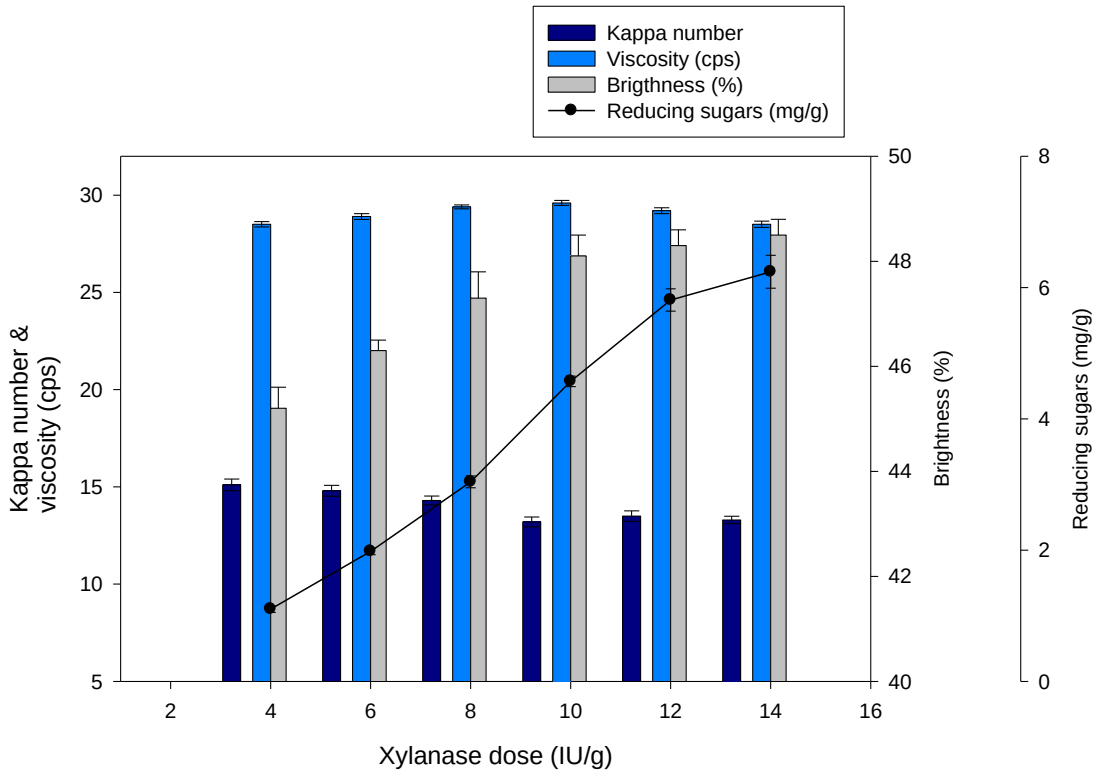

Figure-4.1: Effect of xylanase dose (*A. flavus* ARC-12) on release of reducing sugars, kappa number, viscosity and brightness of pulp

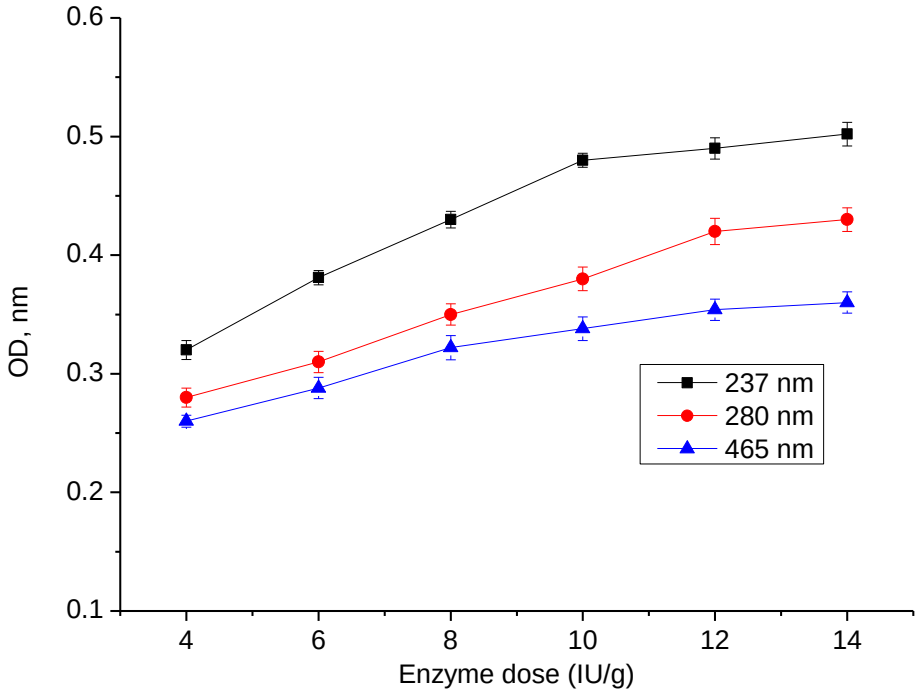

Figure-4.2: Effect of xylanase dose (*A. flavus* ARC-12) on release of chromophores

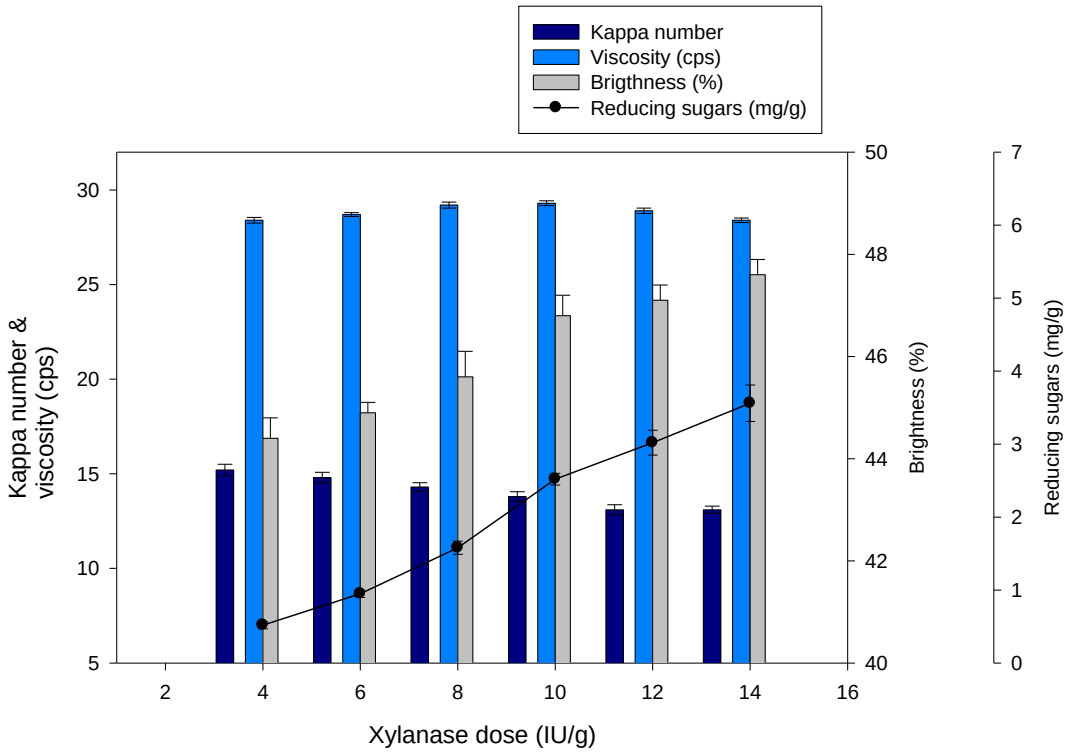

Figure-4.3: Effect of xylanase dose (*S. commune* ARC-11) on release of reducing sugars, kappa number, viscosity and brightness of pulp

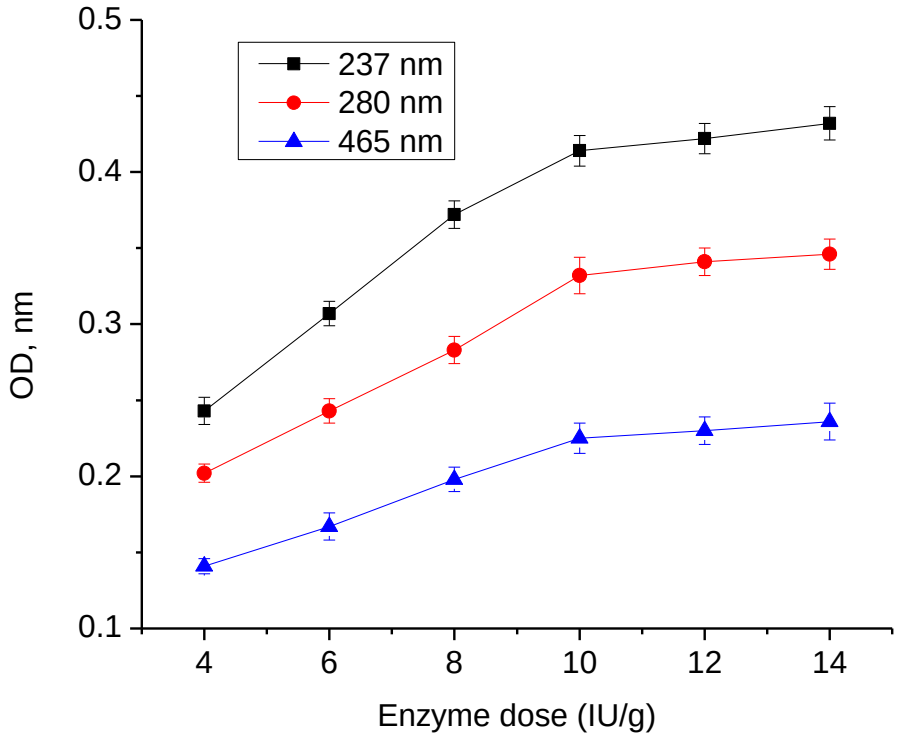

Figure-4.4: Effect of xylanase dose (*S. commune* ARC-11) on release of chromophores

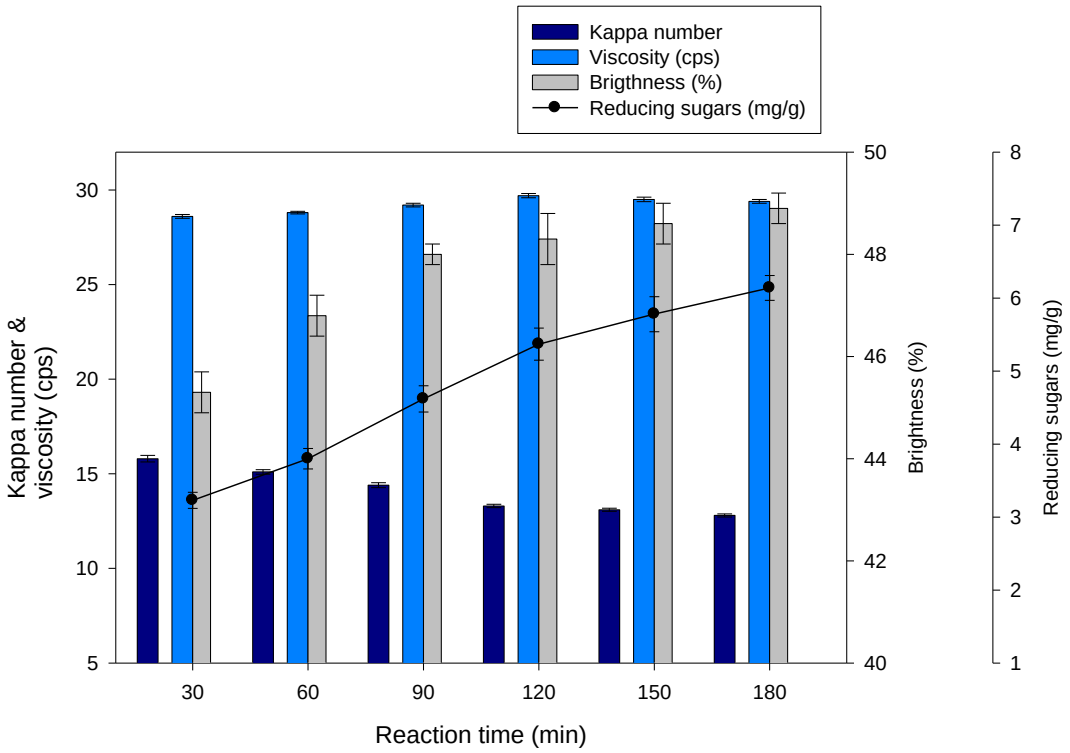

Figure-4.5: Effect of xylanase reaction time (*A. flavus* ARC-12) on release of reducing sugars, kappa number, viscosity and brightness of pulp

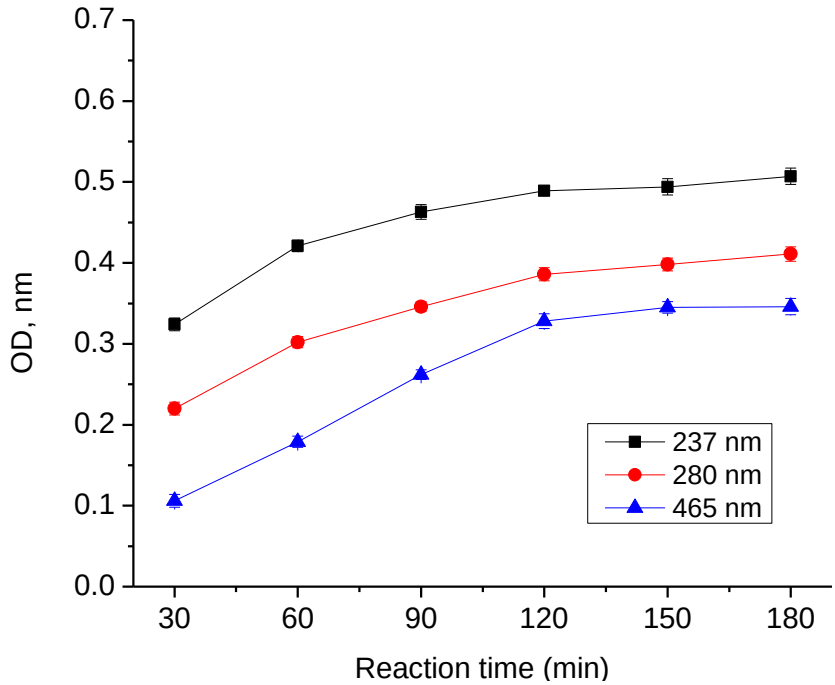

Figure-4.6: Effect of xylanase reaction time (*A. flavus* ARC-12) on release of chromophores

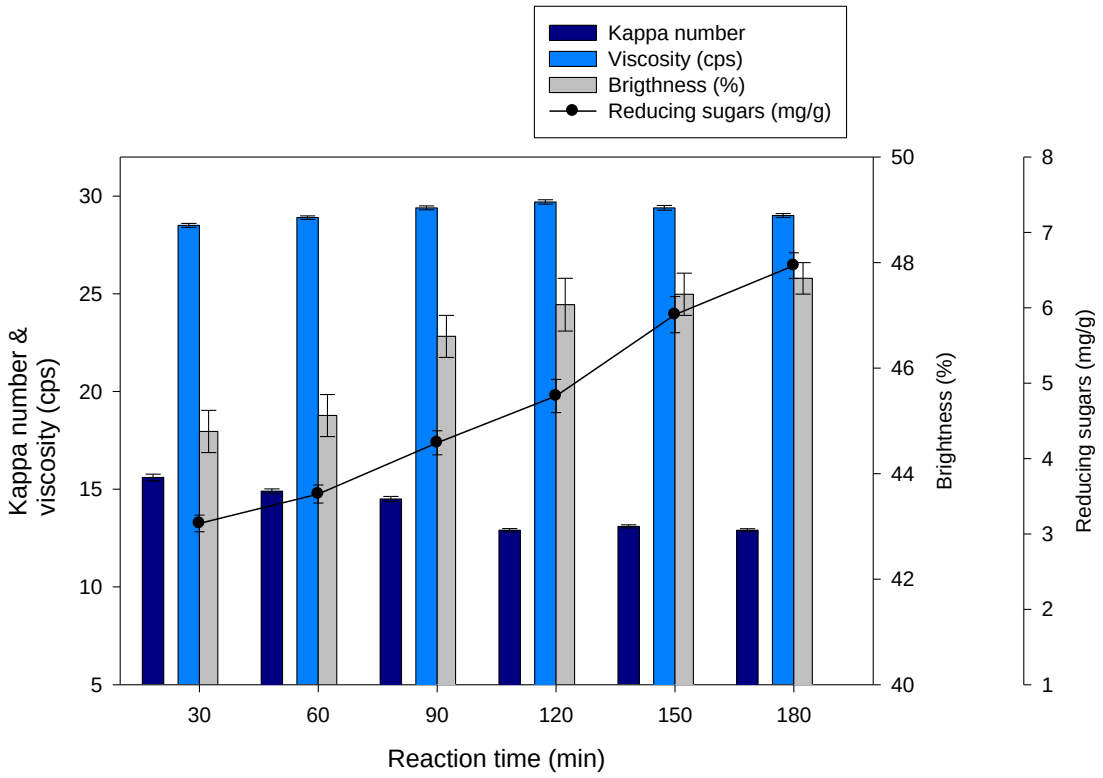

Figure-4.7: Effect of xylanase reaction time (*S. commune* ARC-11) on release of reducing sugars, kappa number, viscosity and brightness of pulp

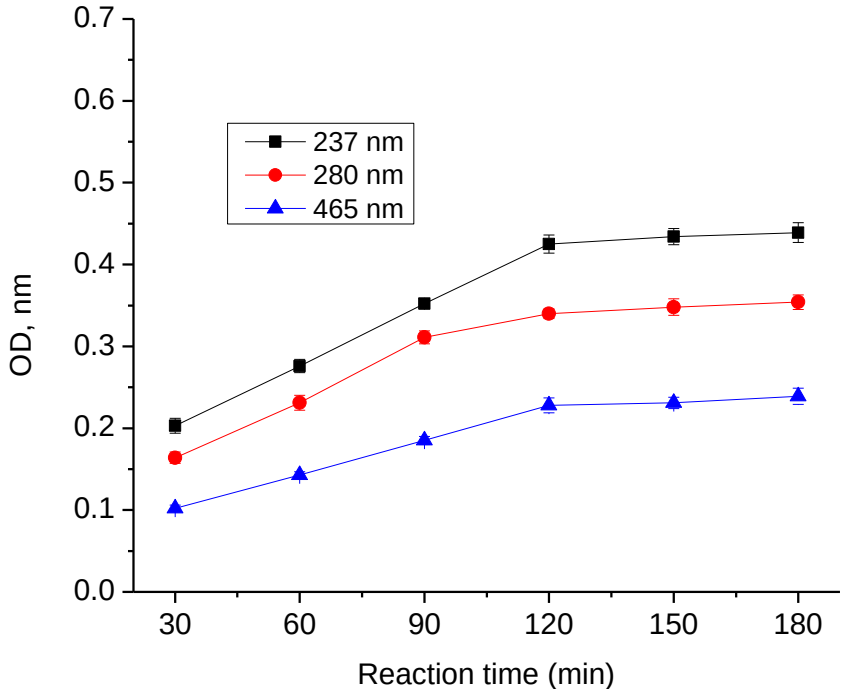

Figure-4.8: Effect of xylanase reaction time (*S. commune* ARC-11) on release of chromophores

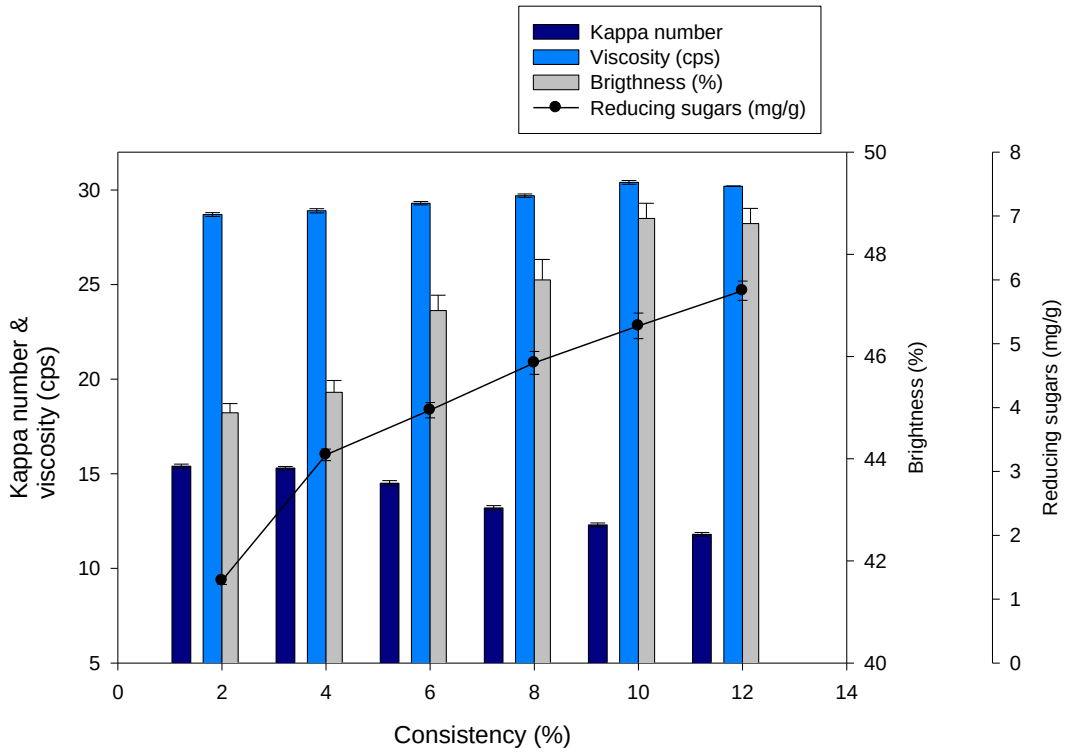

Figure-4.9: Effect of consistency on release of reducing sugars, kappa number, viscosity and brightness of pulp during xylanase pretreatment (*A. flavus* ARC-12)

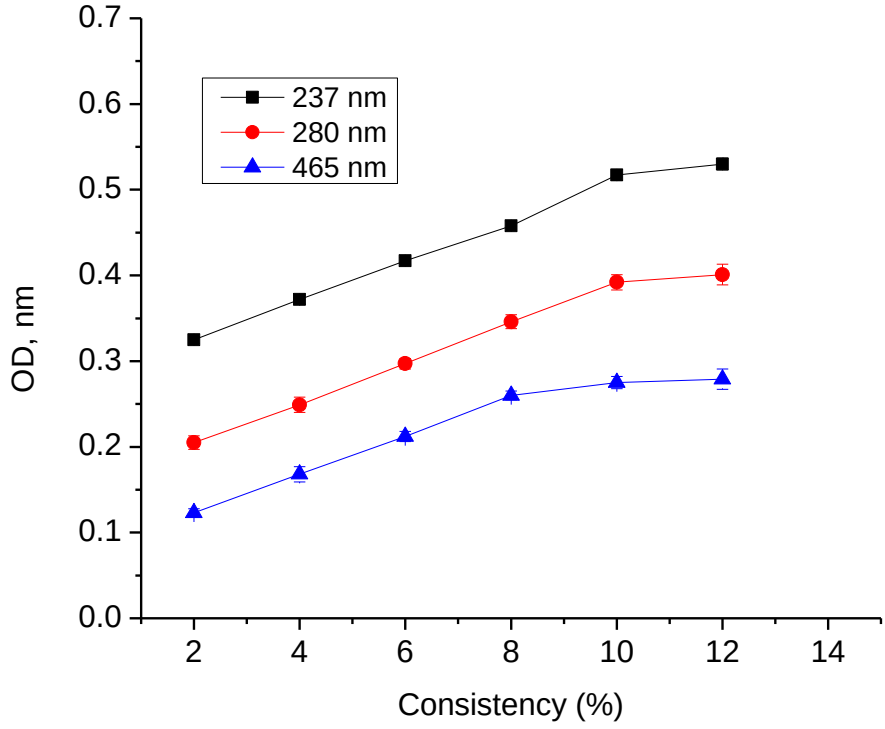

Figure-4.10: Effect of consistency on release of chromophores during xylanase pretreatment (*A. flavus* ARC-12)

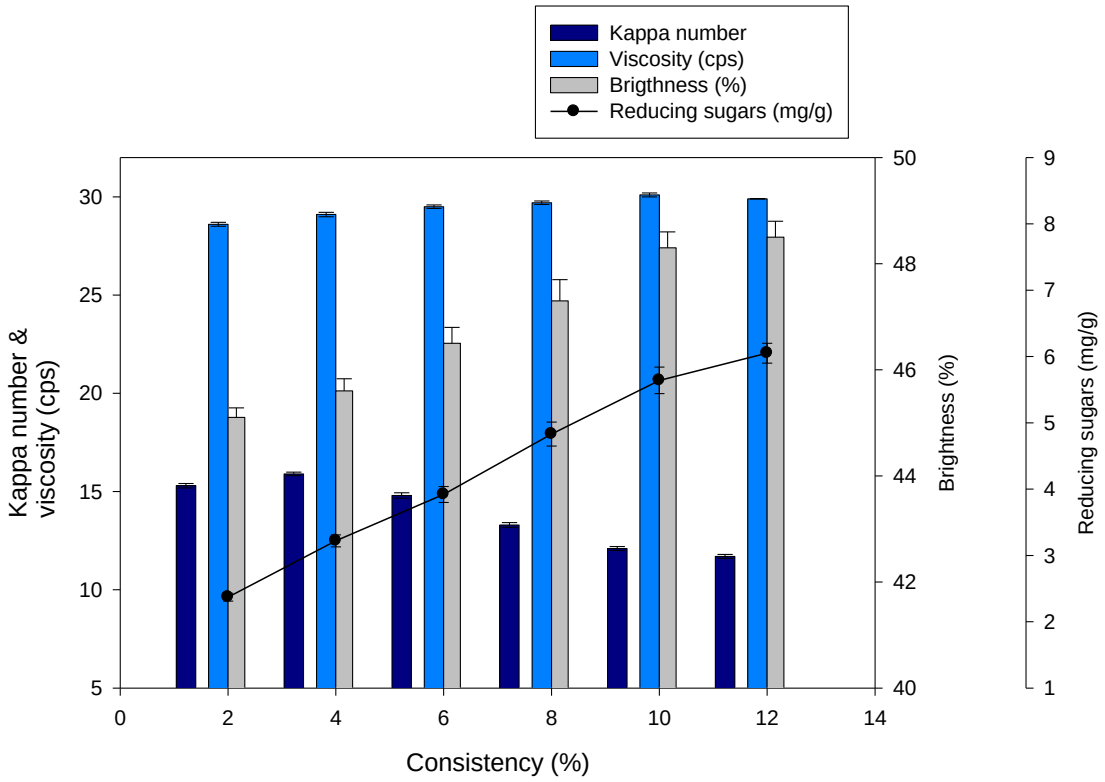

Figure-4.11: Effect of consistency on release of reducing sugars, kappa number, viscosity and brightness of pulp during xylanase pretreatment (*S. commune* ARC-11)

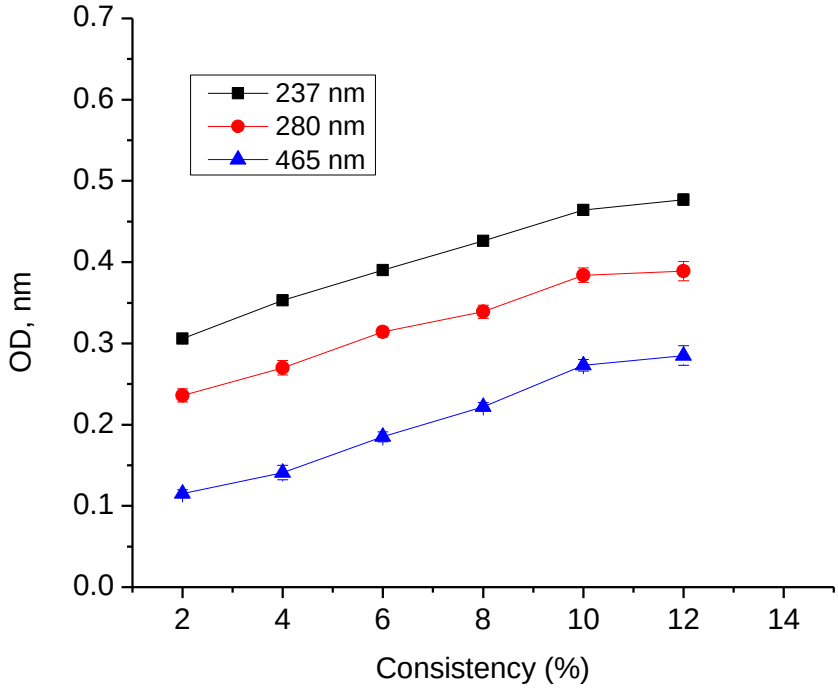

Figure-4.12: Effect of consistency on release of chromophores during xylanase pretreatment (*S. commune* ARC-11)

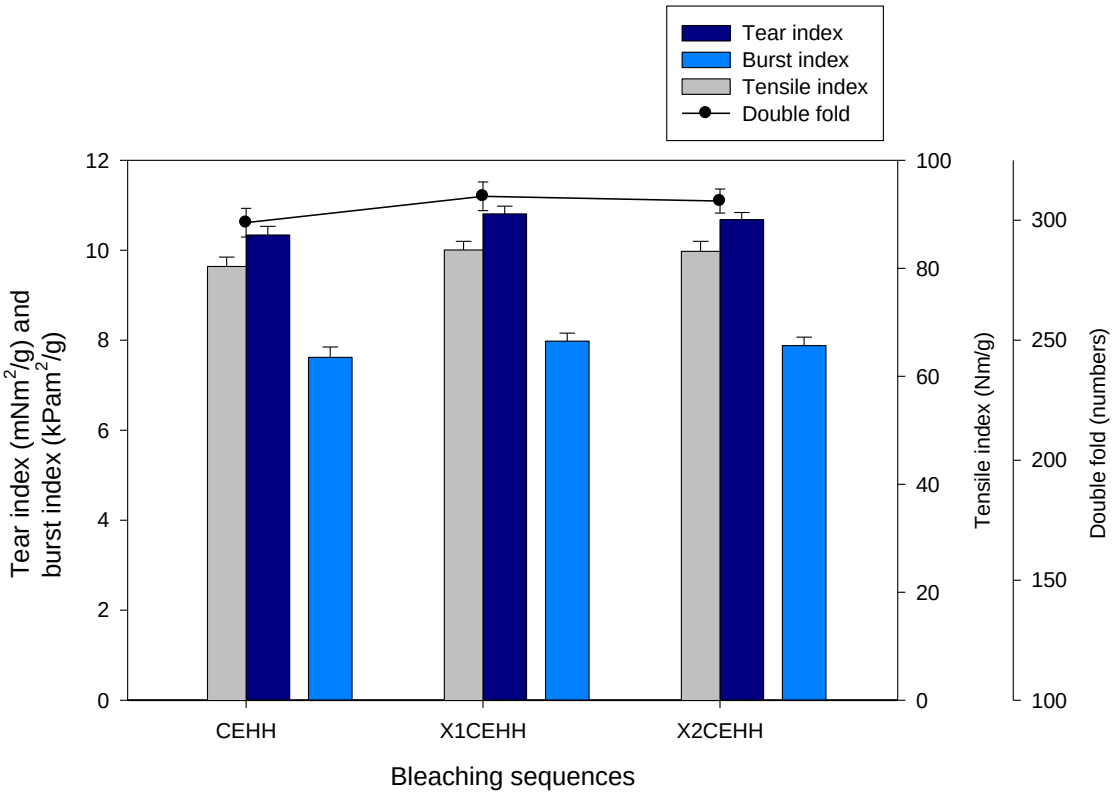

Figure-4.13: Comparison of mechanical strength properties during conventional bleaching of *E. binata* pulp

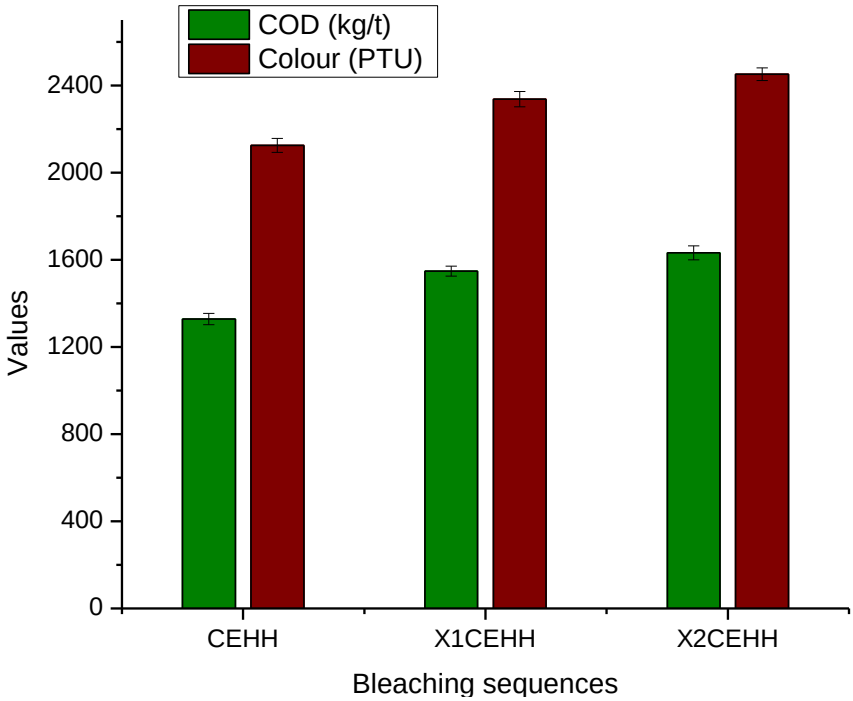

Figure-4.14: Comparison of COD and colour of combined effluent generated during conventional bleaching of *E. binata* pulp

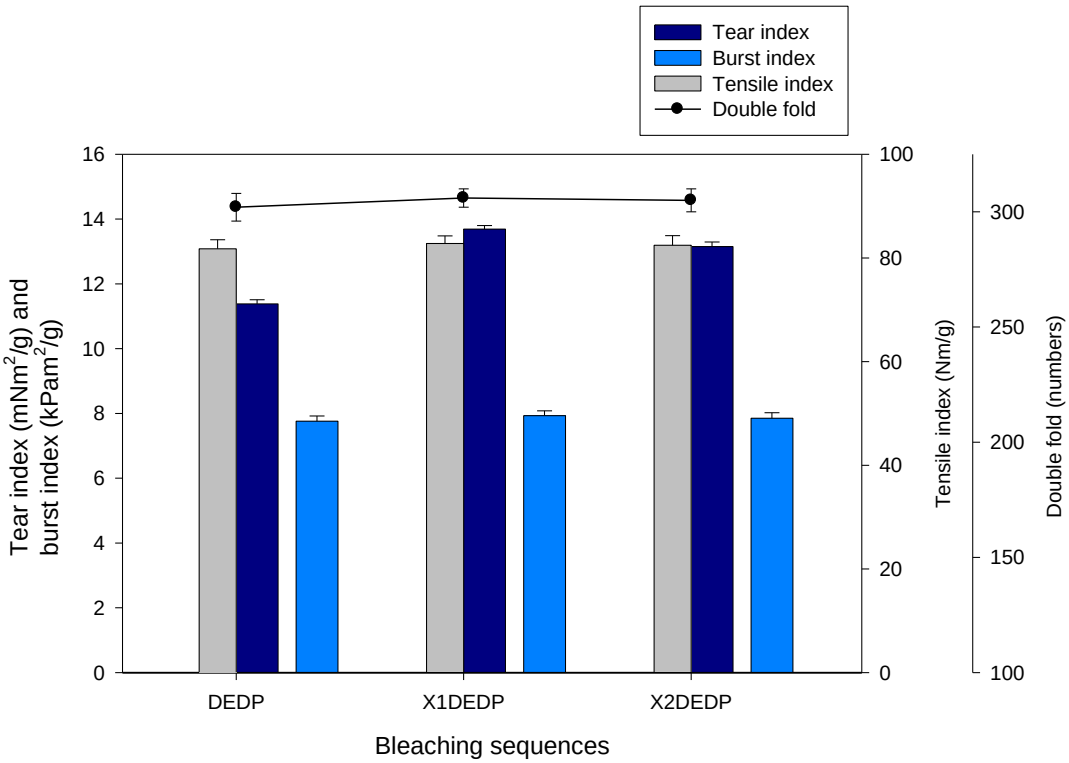

Figure-4.15: Comparison of mechanical strength properties during ECF bleaching of pulp of *E. binata*

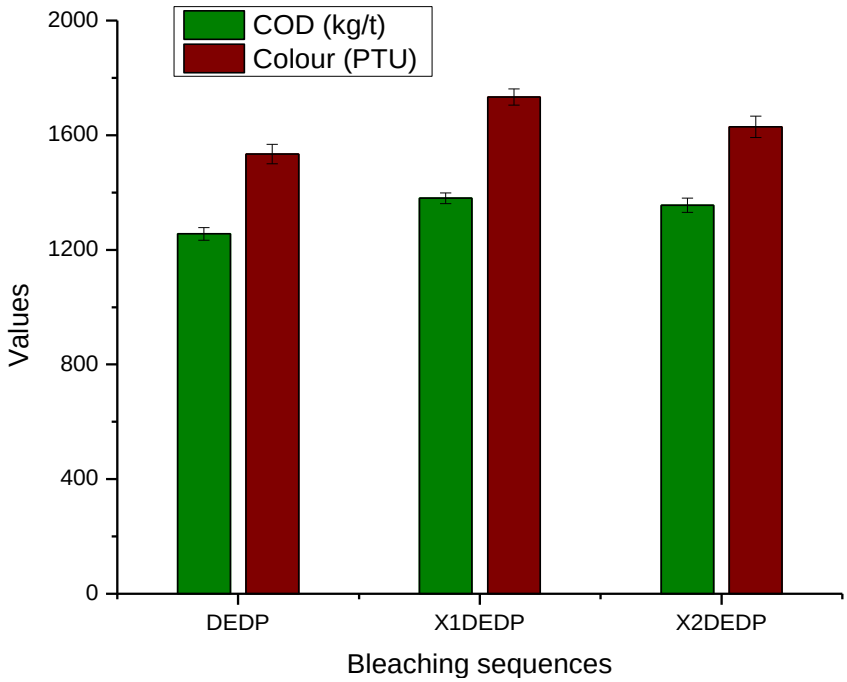

Figure-4.16: Comparison of COD and colour of combined effluent generated during ECF bleaching of *E. binata* pulp

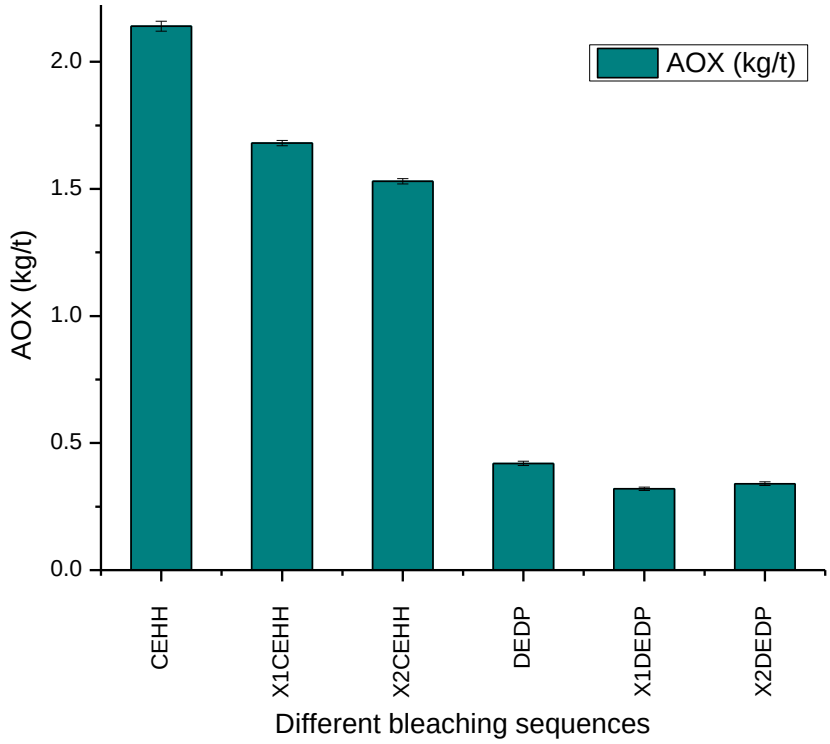

Figure-4.17: Comparison of AOX generated during conventional and ECF bleaching of *E. binata* pulp

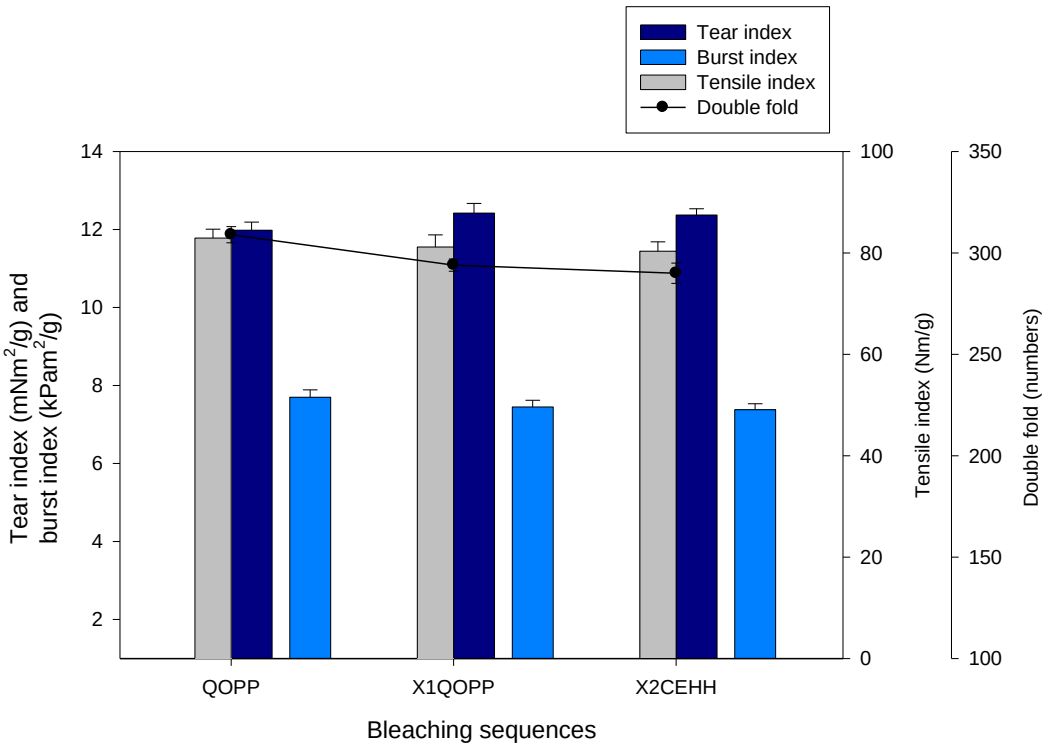

Figure-4.18: Comparison of mechanical strength properties during TCF bleaching of pulp of *E. binata*

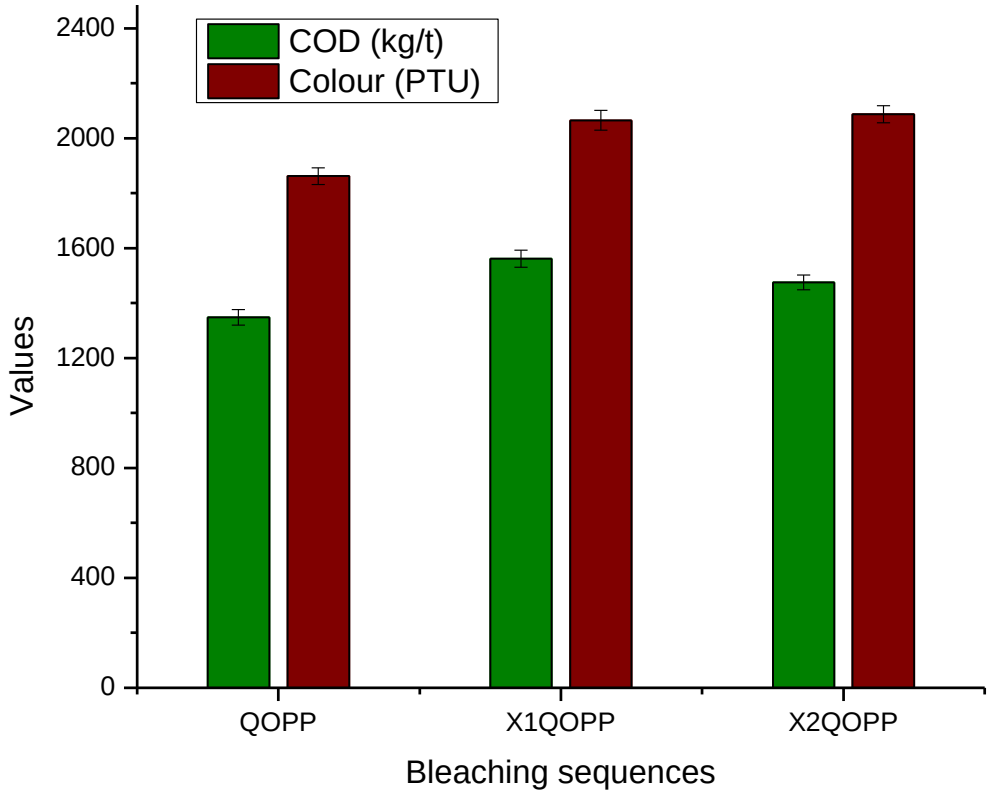

Figure-4.19: Comparison of COD and colour of combined effluent generated during TCF bleaching of *E. binata* pulp

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CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1: Conclusions

Based on present investigations, the following conclusions were drawn:

1. Isolation of xylanolytic fungal strains was carried out from diverse habitats such as manures, dead and decaying wood and soil samples. Two fungal isolates ARC-11 and ARC-12 were selected based on maximum xylanase and minimal cellulase activities. Fungal isolates ARC-11 and ARC-12 were identified as *Schizophyllum commune* and *Aspergillus flavus* respectively based on molecular and morphological characteristics which were designated as *Schizophyllum commune* ARC-11 and *Aspergillus flavus* ARC-12 for further study.
2. The maximum xylanase production from *S. commune* ARC-11 was found with rice straw (4288.36 IU/gds) as the carbon source under SSF conditions. Compared to other agro-residues such as congress grass, wheat bran, maize bran, and rice straw, pearl millet stover showed maximum production of xylanase (1345.44 IU/gds) by *A. flavus* ARC-12. Therefore, rice straw and pearl millet stover was selected for xylanase production by *S. commune* ARC-11 and *A. flavus* ARC-12 respectively.
3. *S. commune* ARC-11 produced maximum xylanase activity (1147.11 IU/ml) and cellulase activity (1.47 IU/ml) at optimum cultural conditions such as incubation time 8th day, temperature 30 °C, pH 7.0, moisture content 70.0%, nitrogen source (ammonium sulphate, 0.08% as available nitrogen), and surfactant (Tween-20, 0.10% (w/v)) using rice straw as the carbon source under SSF conditions. *A. flavus* ARC-12 produced maximum xylanase production (234.26 IU/ml) at optimum cultural conditions such as incubation time 48 h, temperature 30 °C, pH 6.0, moisture content 77.5%, nitrogen source (beef extract, 1.2% (w/v)), and surfactant (Tween-60, 0.10% (w/v)) using pearl millet stover as the carbon source under SSF conditions. Cellulase activity was not detected in the crude enzyme from *A. flavus* ARC-12.
5. Optimum pH for xylanase activities were 5.0 and 6.0 for *S. commune* ARC-11 and *A. flavus* ARC-12 respectively while maximum xylanase activities were observed at temperature 55 and 50 °C for *S. commune* ARC-11 and *A. flavus* ARC-12 respectively.

6. *E. binata* might be used as a blender for other short-fibered raw materials for writing and printing grades due its morphological characteristics such as higher fibre length (2.20 mm), thin walled fibre, higher slenderness ratio (202.76), and other derived values. Proximate chemical analysis of *E. binata* also indicated the suitability of this grass for pulp and paper manufacture. Holocellulose and α-cellulose contents were 73.1% and 46.0% respectively. The higher 1% NaOH solubility (38.0%) of *E. binata* was possibility due to the presence of low molar mass of carbohydrates and other alkali soluble materials. Lower lignin content (21.2%) of *E. binata* was also a favourable factor the removal of lignin during pulping at milder pulping conditions (lower temperatures and chemical charges) to reach a desirable kappa number.
7. During soda-pulping of *E. binata,* maximum pulp yield (43.58%) of kappa number 17.38 with 0.9% screening rejects was obtained at 12% of active alkali (as Na_2O) pulping temperature 140 °C, cooking time 120 min and solid to liquor ratio 1:4.
8. The maximum pulp yield of 47.48% with a kappa number of 16.13 was obtained using 30% ethanol during soda pulping. The pulp yield was improved by 3.9 and 4.72% compared to soda and bio-soda pulping processes respectively while kappa number reduced by 1.25 units compared to soda pulping process.
9. Physical strength properties were improved during ethanol-soda and bio-soda compared to soda pulping. Addition of 30% ethanol during soda pulping of *E. binata* at optimum conditions, improved the pulp brightness by 6.6%, tensile index 32.18%, burst index 35.40% and double fold numbers 77.31% compared to soda pulping. On contrary to this, tear index of ethanol-soda pulp decreased by 9.95% compared to soda pulp. Similarly, bio-soda pulp showed an improvement in tensile index, burst index and double fold numbers by 24.94, 48.45 and 14.03% respectively compared to soda pulp. Following the same pattern, tear index of bio-soda pulp decreased by 12.86% compared to soda pulping.
10. During optimization of prebleaching variables, a xylanase dose of 10IU/g, reaction time of 120 min, and pulp consistency of 10% were found most suitable for prebleaching of ethanol soda pulp of *E. binata* using xylanases (as the prebleacging agent) from *A. flavus* ARC-12 and *S. commune* ARC-11. For *A. flavus* ARC-12, xylanase pretreatment reduced the kappa number by 24.07, improved brightness by 4.8% (ISO) and viscosity by 7.80% for unbleached ethanol-soda pulp of *E. binata*. For *S. commune* ARC-11, xylanase pretreatment

mitigated the kappa number from 25.30%, improved brightness by 4.4% (ISO) and viscosity by 6.73% of unbleached ethanol-soda pulp of *E. binata.*

11. Released chromophores also indicated the enzyme attack on the pulp as oligosaccharides were released by the initial de-polymerization of the xylan coating on the fibre surface.
12. Xylanase pretreatment followed by alkali extraction declined the total chlorine demand by 23.5 and 24.5% for ethanol-soda pulp of *E. binata* during X_1ECEHH and X_2ECEHH. AOX were mitigated by 21.49 and 28.50% during X_1ECEHH and X_2ECEHH bleaching sequences respectively compared to CEHH. The brightness of ethanol-soda pulp of *E. binata* bleached by X_1ECEHH and X_2ECEHH bleaching sequence improved by 1.6 and 0.8% (ISO) respectively along with slight improvement in physical strength properties compared to CEHH bleaching sequence. Pulp viscosity of ethanol-soda pulp of *E. binata* bleached by X_1ECEHH and X_2ECEHH bleaching sequence improved by 4.76 and 3.57% respectively compared to CEHH bleaching sequence. COD load in combined bleach effluent was increased by 16.56 and 22.89% and colour was increased by 10.02 and 15.39% respectively during X_1ECEHH and X_2ECEHH bleaching sequences respectively compared to CEHH.
13. Brightness and viscosity of DEDP ethanol-soda pulp improved by 1.4% (ISO) and 4.76% respectively compared to CEHH bleaching sequence. The combined effluent generated during DEDP bleaching sequence of ethanol-soda pulp of *E. binata* showed a decrease in COD by 5.42%, colour by 27.77%, and AOX by 80.37% respectively compared to CEHH bleaching sequence. The brightness of X_1DEDP, and X_2DEDP bleached ethanol-soda pulp of *E. binata* increased by 3.2 and 1.9% (ISO) respectively compared DEDP bleaching sequences at the same chlorine dioxide charge. The viscosity of X_1DEDP, and X_2DEDP bleached *E. binata* ethanol-soda pulp increased by 3.40 and 2.27% respectively compared to DEDP bleaching sequence. The combined effluent generated during DEDP bleaching sequence of ethanol-soda pulp of *E. binata* showed a decrease in AOX by 80.37% compared to CEHH bleaching sequence. Xylanase pretreatment reduced AOX generation by 23.80 and 19.04% after X_1DEDP, and X_2DEDP bleaching sequences respectively compared to DEDP.
14. Brightness of *E. binata* ethanol-soda pulp was increased by 2.5 and 1.4% (ISO) as well as pulp viscosity by 3.33 and 5.55% during X_1QOPP and X_2QOPP bleaching sequences respectively compared to QOPP bleaching sequence. Tear index increased by 3.67 and 3.25% while other physical strength properties such as burst index, tensile index and double fold number were decreased slightly during X_1QOPP and X_2QOPP bleaching sequences

compared to QOPP. The COD values were increased by 15.87 and 9.42% and colour of combined bleach effluent generated increased by 10.90 and 12.08% X_1QOPP and X_2QOPP bleaching sequences respectively compared QOPP bleaching sequence.

15. Finally it can be concluded that ethanol-soda pulping process for *E. binata* was suitable to minimize carbohydrate degradation compared to other conventional pulping methods suitable for pulping of other non-woods or grasses. Similarly, all the three major bleaching processes like conventional, ECF and TCF bleaching sequences were studied and effect of enzymes on pulp yield, optical properties, mechanical strength properties and effluent characteristics were studied. Xylanases from *A. flavus* ARC-12 and *S. commune* ARC-11 had tremendous potential not only for reducing the bleach chemical demand and toxicity of various bleaching effluents in terms of AOX but also for improving or maintaining some of the paper properties. Hence, the study brought about for the development of sequences that would be environment friendly in terms of AOX generation for ethanol-soda pulps of *E. binata*, indicating the effectiveness of xylanase biobleaching for improving environmental performance of the bleach plant.

5.2: Future recommendations

With reference to present work done, some important suggestions have been drawn for future study. The suggested work for future could not be done due to time constraints and limitation of the work plan for PhD studies. The following suggestions are made for the future work:

1. Xylanase production by fungal strains *S. commune* ARC-11 and *A. flavus* ARC-12 further can be improved by using response surface methodology.
2. Further, purification of enzymes is recommended for further characterization of enzymes and better understanding of their complex enzyme system and function of individual component of enzyme during enzymatic deinking mechanism. Purified enzymes may also be subjected to enzyme kinetics parameters.
3. Xylanases from fungal strains *S. commune* ARC-11 and *A. flavus* ARC-12 may be effective for several other industrial applications such deinking of waste paper, and hydrolysis of xylan during bioconversion processes.
4. A plant trial can be conducted with xylanases from *S. commune* ARC-11 and *A. flavus* ARC-12 in a non-wood fibre-based pulp and paper industry using *E. binata* as the raw material to validate laboratory results and cost reduction studies must be carried out to calculate the

economic viability of crude enzyme. Xylanases from *S. commune* ARC-11 and *A. flavus* ARC-12 can be evaluated for prebleaching of different cellulosic raw materials.

5. Chromatographic analysis can be carried out to study the change in molecular weight profiles of lignin and carbohydrates in the pulp filtrate for a better understanding of the attack of xylanase on lignin-carbohydrate complexes (LCC).

Publications

1. Archana Gautam, Amit Kumar and Dharm Dutt (2015) Production of cellulase-free xylanase by *Aspergillus flavus* ARC-12 using pearl millet stover as the substrate under solid-state fermentation**,** Journal of Advanced Enzyme Research, 1, 1-9.

2. Archana Gautam, Amit Kumar and Dharm Dutt (2016) Effects of Ethanol Addition and Biological Pretreatment on Soda Pulping of *Eulaliopsis binata*, Journal of Biomaterials and Nanobiotechnology, 7 (2), 78-90.

3. Archana Gautam, Amit Kumar and Dharm Dutt, Production and characterization of cellulase-free xylanase by Aspergillus flavus ARC-12 and its application in pre-bleaching of ethanol-soda pulp of *Eulaliopsis binata*, Research Journal of Biotechnology (Communicated).