STUDIES ON ALKALIPHILIC CELLULASE PRODUCING FUNGI

A Thesis

Submitted in partial fulfillment of the requirements for the

Award of the degree of

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

By

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Supervised By

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LOVELY PROFESSIONAL UNIVERSITY
PUNJAB
2020

DECLARATION

I hereby declared that the work for the thesis entitled, **"Studies on alkaliphilic cellulase producing fungi"** submitted to S**chool of Bioengineering and Biosciences,** Lovely Professional University, Phagwara for the award of degree of Doctor of Philosophy (Microbiology) is entirely my own work and has not been submitted in part or full for any other degree/diploma at this or any other University/Institution. All the ideas and references have been duly acknowledged.

ABDULHADI YAKUBU

(11717810)

CERTIFICATION

It is hereby certified that the work for the thesis entitled **"Studies on alkaliphilic cellulase producing fungi"** submitted to S**chool of Bioengineering and Biosciences,** Lovely Professional University, Phagwara, for the award of degree of Doctor of Philosophy (Microbiology) was carried out in the Department of Microbiology by **Abdulhadi Yakubu** under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation. No part of this thesis has ever been submitted for any other Degree/Diploma at this or any other University/Institution. The thesis is fit to be considered for the award of degree of Ph.D.

Dr. ASHISH VYAS
(SUPERVISOR)

ABSTRACT

Alkaliphilic cellulase producing fungi were isolated from alkaline soil and industrial effluents from two districts (Kapurthala and Amritsar) of Punjab region, India. Mandel and Reese media supplemented with Carboxymethyl Cellulose (CMC) and cellulose powder (CP) under different pH values (7-11) was used for isolation and screening. Twenty seven (27) alkaline fungal cultures were isolated from seventeen (17) different samples. Seven fungal cultures (VSTPDKF2, VSTPDKF1, VRTPDKF1, VNTPDKF2, KPMAS2F1, KPMAS4F1 and WSSDBS2F1) were found to have the positive cellulase enzyme activity in Congo Red screening. These seven alkaline cellulase fungi were subjected to endoglucanase and exoglucanase (Enzyme assay) production using Dinitrosalicyclic acid (DNSA) method at different incubation days (4^{th} -12^{th}), 30^{O}C temperature and pH 8. All the fungal cultures were found to have an optimum enzyme activity on 8^{th} day incubation following which the activity declined. Four of these cultures with highest enzyme activity (VSTPDKF2, VSTPDKF1, VRTPDKF1 and WSSDBS2F1) were selected and identified as genera of *Fusarium* (VSTPDKF2), *Aspergillus* (VSTPDKF1), *Penicillium* (VRTPDKF1) and *Mucor* (WSSDBS2F1) based on their colony appearance and microscopic examination. Following morphological and microscopic examination, these four alkaline fungal genera were further identified up to species levels as *Fusarium oxysporum* VSTPDKF2, *Aspergillus oryzae* VSTPDKF1, *Penicillium chrysogenum* VRTPDKF1 and *Mucor circinelloides* WSSDBS2F1 when Integrated Transcribed Spacer TS1 and ITS4 (ITS1 5′-GTAGTCATATGCTTGTCTC-3′) and (ITS4 5′-CTTCCGTCAATTCCTTTAAG-3′) primers were amplified by 18S rRNA gene region, sequenced, analyzed and BLAST for similarity among species in NCBI data base.

Optimization for the production of endoglucanase (CMCase) and exoglucanase (FPase) from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 using One Factor at a Time (OFAT) approach as well Central Composite Design (CCD) through Response Surface Methodology (RSM) approach were conducted. The pH, temperature, incubation time, lignocellulosic carbon source, nitrogen source and ammonium sulphate concentration are the physico chemical and nutritional parameters used for the production of alkaline cellulase based on OFAT. Maximum cellulase activity from *F. oxysporum* VSTPDKF2 in both CMCase and FPase (3.330 IU/ml and 3.89 FPU/ml) and *M. circinelloides* WSSDBS2F1 in both CMCase and

FPase (2.78 IU/ml and 3.33 FPU/ml) were reported at neutral pH. However, significant amount of enzyme was produced across alkaline environment (8-10). Optimum temperature for both *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 on CMCase (3.50 IU/ml and 3.32 IU/ml) and FPase (3.70 FPU/ml and 3.52 FPU/ml) was found at $30^{O}C$ respectively. Incubation time was observed that at 8^{th} day with optimum CMCase and FPase activity from *F. oxysporum* VSTPDKF2 and *Mucor circinelloides* WSSDBS2F1 were (3.52 IU/ml and 3.88 FPU/ml) and (2.78 IU/ml and 3.88 FPU/ml) respectively. Alkali pre treated Rice straws (RS), wheat straw (WS) and sugarcane bagasse (SB) were used as carbon source in place of CMC and cellulose powder in the production medium. Maximum CMCase (2.78 IU/ml) and FPase (3.70 FPU/ml) activity was obtained on rice straw from *F. oxysporum* VSTPDKF2 as well as CMCase (3.70 IU/ml) and FPase (3.89 FPU/ml) activity on sugarcane bagasse from *M. circinelloides* WSSDBS2F1 were reported. Ammonium sulphate (NH_4SO_4) was found to have maximum enzyme activity of CMCase (3.50 IU/ml) and FPase (3.70 FPU/ml) from *F. oxysporum* VSTPDKF2. *M. circinelloide*s WSSDBS2F1 has shown an optimum CMCase (3.52 IU/ml) and FPase (3.50 FPU/ml) respectively when four different inorganic nitrogen sources which includes ammonium sulphate (NH_4SO_4), ammonium carbonate (NH_4HCO_3), ammonium chloride (NH_4Cl) and Sodium nitrate ($NaNO_3$) were used as source of nitrogen.

The pH (A), temperature (B) and concentration of ammonium sulphate (C) were selected for optimizing cellulase production using Central Composite Design (CCD) after One Factor at a Time (OFAT) approach. Twenty (20) different predicted designed experiments each from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 for endoglucanase and exoglucanase activity were conducted in the laboratory to determined the actual enzyme activity of each experiment. An estimated endoglucanase and exoglucanase as a function of independent variables were obtained after ANOVA has provided a regression equation. The coefficient (R^2) values of the experiments are close to one (1) which signified that the model behavior can be interpreted for alkaline enzyme production. The Adjusted R^2 from this model was 0.9873 and 0.9798 as well as 0.9721 and 0.9808 for the two fungal isolates respectively. This is an indication of good relationship between actual and predicted values. Adequate precisions of the model measured the signal to noise ratio for CMCase and FPase from *F. oxysporum* VSTPDKF2 (48.790 and 39.373) and *M. circinelloides* WSSDBS2F1 (31.58 and 36.622) respectively indicated an adequate signal. The result also indicated a high models *F*-value for alkaline

CMCase and FPase produced from *F. oxysporum* VSTPDKF2 as 165.31 and 103.28 with their respective small prob >F values (P-value) of <0.0001 that signify the models were significant. Meanwhile, CMCase and FPase produced from alkaline *M. circinelloides* WSSDBS2F1 shows an *F*-value of 74.56 and 109.01 respectively with their corresponding prob >F values (P-value) of <0.0001 indicated that that the model is significant. The lack of fit F-value of the models for CMCase and FPase from alkaline *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 with their corresponding lack of fit P-value indicated that lacks of fits were not significant and that models are very accurate without any noise. To examine the significance of the facts, validation the experimental design between RSM-CCD and OFAT results was conducted. RSM experiment from *F. oxysporum* VSTPDKF2 reported an actual enzyme activity of 3.91 IU/ml and 4.26 FPU/ml for CMCase and FPase respectively. Similarly, *M. circinelloides* WSSDBS2F1 has reported actual enzyme activity from both CMCase and FPase as 4.26 IU/ml and 4.26 FPU/ml respectively. These experiments were conducted based on the different predicted experiments from three factors identified as pH, temperature and NH_4SO_4 concentration in order to validate the results previously conducted with CCD. Comparison between OFAT and RSM from *F. oxysporum* VSTPDKF2 revealed an increased enzyme activity of 0.39 IU/ml (CMCase) and 0.19 FPU/ml (FPase) using RSM as compared to that of OFAT. Similarly, *M. circinelloides* WSSDBS2F1 CCD result revealed an increased enzyme activity of 0.74 IU/ml and 0.56 FPU/ml for CMCase and FPase respectively as compared to OFAT.

Alkaline endoglucanase from both *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 were evaluated for their deinking potentials. An A4 paper was printed with word ‘deinking’ for approximately 640 words using xerox printer. After enzymatic, chemical and control pulping process as well as flotation, the resultant pulp was prepared according to TAPPI standard and sent to the Central Pulp & Paper Research Institute (CPPRI) Saharanpur, 247001 (U.P.) India for the testing of pulp brightness, tensile strength, bursting strength as well as tearing strength. The percentage brightness result following *F. oxysporum* VSTPDKF2 enzymatic treatment indicated an improvement in ISO brightness of 2.36% and 0.48% on control and chemical respectively from the prepared hand sheet. Moreover, 1.00% and 0.12% improvement in ISO brightness were recorded following enzymatic treatment of CMCase from *M. circinelloides* WSSDBS2F1. Enzymatic deinking using CMCase produced from *F. oxysporu*m VSTPDKF2 and *M. circinelloides* WSSDBS2F1 improved the tensile strength of the

printed waste paper by 10N/m and 127N/m as well as 180N/m and 253N/m respectively when compared to control and chemical deinking process. In terms of bursting strength, enzymatic deinking of the enzyme produced by these isolated fungi increase the bursting strength of the deink hand sheet by 3KPa and 1KPa from *F. oxysporu*m VSTPDKF2 and also 11KPa and 8KPa from *M. circinelloides* WSSDBS2F1 when compared to control and chemical deinking respectively. The tearing strength shows an improvement of 3mN and 9mN as well as 11mN and 8mN following enzymatic deinking alkaline CMCase isolated from *F. oxysporum* VSTPDKF1 and *M. circinelloides* WSSDBS2F1 respectively as compared to control and chemical deinking process respectively. In order to confirm the influence of these enzymes for potential deinking ability, the prepared pulp was subjected to Scanning Electron Microscope (SEM). Strength improvement of internal and surface fibrillation of the hand sheet can be clearly seen in different magnification of Scanning Electron Microscope (SEM) as compared to chemically deink and control untreated pulp. Fiber swelling, loosing and peeling off of fiber are clearly visible on enzymatic deink pulp when compared with control and chemically deink pulp. A Fourier Transform Infrared Spectroscopy (FTIR) was also conducted on enzymatic, chemical and control deink hand sheets in order to examine the surface functional groups of the pulp. The FTIR spectra of chemical, control and enzyme deink pulp along with their control were recorded over a range of 4000-400cm^{-1} using 25 scans per sample at resolution of 4cm^{-1}. There was clear peak of the hydroxyl group (-OH) of cellulose between 3333cm^{-1} to 3335cm^{-1} for all samples but with different intensity of 93.67%, 88.64% for two enzyme deinking pulp as compared to 68.28% and 69.67% for chemically and control treated pulp respectively. This increase in percentage intensity after enzymatic treatment is an indication of increase in cellulosic content of the pulp. Other areas also indicated similar changes in the enzyme treated pulp as compared to chemical and control treated hand sheets.

Dedication

This PhD work is dedicated to:

- **My late father, may his gentle soul rest in peace,**
- **My mother,**
- **Wife and**
- **Children**

ACKNOWLEDMENT

Firstly, all thanks goes to the Almighty ALLAH for giving me the strength and opportunity to complete this research work.

I would like to extend profound gratitude to my supervisor Dr. Ashish Vyas, Professor and Head, Department of Microbiology and Biochemistry, Lovely Professional University for his guidance, resilience, dedication and total support from the beginning to the end of this research work. He sacrificed his vital time (at home and in office) in guiding me throughout these years despite his tied administrative schedules. May almighty reward you abundantly.

I would also like to use this opportunity to thanks the entire faculty members of the School of Bioscience and Bioengineering most especially our very active head of school in person of Dr. Neeta Raj Sharma who always try her best to see that we have all the necessary material, teaching and Laboratory support throughout these years of research. My appreciation also goes to the head of Laboratory in person of Dr. Chirag Chopra and his wife Dr. Reena Singh Chopra for their guide and support. Also thanks to Dr Gaurav Kumar for his constant advice whenever needed. I also want to thank the entire Lab assistance most especially Kuldeep Singh, whom we stay in our research Lab for sometimes and providing me all necessary support wherever required at any stage of my project. Also want to pay special thanks to my friends especially Rabiu Garba Ahmad, Salim Farouk Bashir, Daljit Singh, Bhupender, Kamaldeep Singh Kaur whom we shared a lot of scientific knowledge throughout my research work.

To my parent, wife (Zainab) and children (Ahmad, Khalifa and Haneef), I would to say a big thank you for your patient, prayer and support while away from you during these years of my research. Thanks also goes to the rest of my family and friends at home that are too numerous to mention. Lastly, I would like thank my institution **(JIGAWA STATE POLYTECHNIC DUTSE)** by approving and sponsoring my PhD research work through a grand from **Tertiary Education Trust Fund (TETFUND)**.

(ABDULHADI YAKUBU)

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CHAPTER ONE

1.0 Introduction

Cellulose is considered as one of the polysaccharides abundant on earth, consisting mainly of D-glucose subunits connected by β-1, 4 glycosidic bonds **(Fig. 1.1.0)**. Fungi and bacteria are known mainly for bioconversion of cellulose to fermentable sugars in a process called saccharification. Microorganisms may use certain sugars released for growth and development by the production of cellulase enzyme which catalyzed the hydrolysis process. Plant biomass is a term which refers to all organic materials found on earth, including lignocellulose, starch and sugar (McKendry, 2002). Among this biomass, lignocellulose was composed of three sugars that consist of cellulose (50%), hemicellulose (30%) and lignin (20%) (Coral, *et al*., 2002). Various types of microorganisms particularly bacteria and fungi are responsible for the degradation and utilization of lignocellulosic biomass to generate carbon as an energy source (Sánchez, 2009). However, fungi are known to be the main organism responsible for the degradation of lignocelluloses resulting in two extracellular enzymes. The hydrolysis of this biomass into fermentable sugars by certain lignocellulolytic enzymes accompanied by fermentation of these sugars to different products is mainly carried out by bioconversion of lignocellulolytic materials (Sánchez, 2009; Sajith, *et al*., 2015).

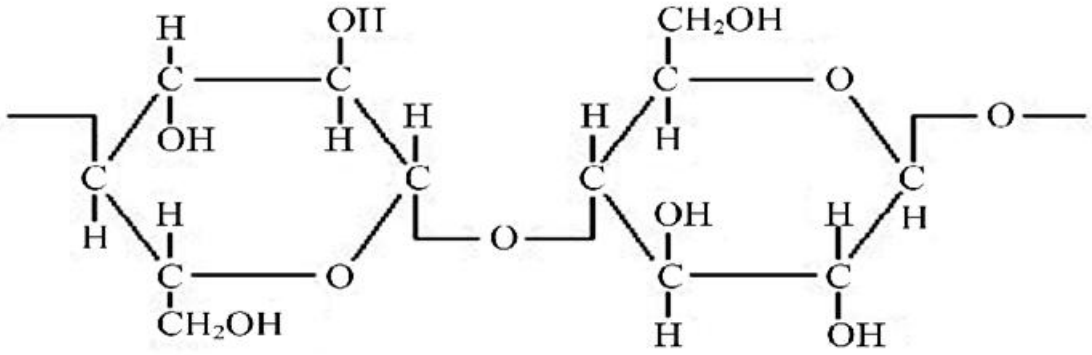


Figure 1.1.0: A structure of cellulose

Despite the fact that many microorganisms are capable of degrading lignocelluloses as carbon and energy sources, only a few are able to generate all the enzymes capable of degrading it into simple monosaccharide sugars necessary for their aerobic or anaerobic metabolic activities (Dashtban *et al*., 2009). As part of their natural ecological recycling, bacteria and fungi can breakdown lignocellulolose with a complex collection of enzymes that includes cellulase, hemicellulase and ligninase. In this case, enzyme ligninolytic enzymes consist of ligninase, laccases, peroxidases and oxidases which breakdown lignin into low molecular weight compound. Fungi such as *Aspergillus nidulans, Phanerochaete chrysosporum* are known to generate laccases enzyme (Arora and Sharma 2010). Hemicellulases on another hand, consist of carbohydrate esterase such as xylanases, (EC 3.2.1.8), β-mannanases (EC 3.2.1.78), arabinofuranosidases (EC 3.2.1.55) and β-xylosidases (EC 3.2.1.37). Xylanase enzyme breakdown hemicelluloses which contain around 70% xylan into oligomers which were subsequently degraded by β-xylosidases into xylose (Dashtban *et al.,* 2009; (Banerjee *et al.,* 2010). However, the third and most common lignocellulolytic enzyme is the cellulase which is an essential enzyme for bioconversion of cellulose to monosaccharides (Hofrichter, 2002). Cellulase is a family of three (3) groups of enzymes called endo-(1,4)-β-D-glucanase (EC3.2.1.4), exo-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). The endoglucanase (EG) target internal O-glycosidic bonds spontaneously, resulting in glucan chains of different lengths; exoglucanase (CBH) on the other acts on the ends of the cellulose chain and releases β-cellobiose as the end product; whereas the β-glycosidases act specifically on the β-cellobiose disaccharides and generate glucose (**Figure 1.2**). Microbial cellulase consists of either domains or modules that are independently folding, structurally and functionally discrete components. Many microorganisms such as bacteria and fungi produce these cellulase enzymes when they grow on cellulosic materials. These microorganisms can either be aerobic, anaerobic, alkaliphilic, mesophilic or thermophilic among which includes the genera of *Clostridium, Cellulomonas, Thermomonospora, Trichoderma, Fusarium, Mucor* and *Aspergillus* are the most extensively studied cellulase producers (Kikani *et al.,* 2010).

Alkaliphilic microorganisms or alkaliphiles are organisms that grow best at pH values exceeding pH 9, usually in the 10–13 range of pH. These include obligate alkaliphiles that can grow only at pH values between pH 9 and above and facultative alkaliphiles that grow optimally at high alkaline conditions but can also grow near neutral pH. Alkaliphilic microorganisms are an important source of useful, stable enzymes among which includes cellulases, hemicellulases, xylanase, esterase, pectinase and amylase (Kumar *et al*., 2018; Abo-State *et al.,* 2013; Kalpana and Rajeswari 2015; Adhyaru *et al.,* 2017; Wang *et al*., 2018). They have a wide range of ecological niches ranging from alkaline soda lakes (Nyakeri *et al.,* 2018) and soils (Vega *et al*., 2012; Kim, *et al*. 2012) that are subjected to ammonification and human industrial processes generating high pH. An excellent industrial enzyme is expected to possess high stability and activity in a wide range of fermentation conditions. These types of enzymes are mainly found in extreme environments such as hot spring (thermophiles), Antarctic seawater (psychrophiles), deep-sea hydrothermal vents (barophiles), alkaline soda lake (alkalophiles), hot sulphuros springs (acidophiles) and natural/artificial salts (halophiles) Kladwang *et al*. (2003). Alkaline cellulase has different biotechnological applications in industries ranging from paper, textile, food, detergent and biofuel (Sajith *et al*., 2015). Despite these enormous applications, the industrial uses of these enzymes are affected by lack of microbial strains capable of generating a high amount of alkaline cellulase (Paul *et al*., 2017).

Industrial utilization of waste papers in the production of a new one is increasing globally. Currently, the pulp and paper industry is one of the largest consumers of wood. Based on their demands due to global economic growth, more trees will be harvested and waste will be consuming and dispose of in the environment (Pathak *et al*., 2011). Chemical agents such as sodium hydroxide, hydrogen peroxide, sodium carbonate, diethylenetriaminepentacetic acid, sodium silicate and surfactants are used in a large quantity by paper industries as conventional methods of deinking waste paper which lead to expensive wastewater treatment to meet environmental regulations (Saxena and Chauhan 2016). Enzymes such as lipase, xylanase, pectinase, cellulase, hemicellulase,

amylase, and esterase are used as a substitute to chemical conventional methods of deinking waste papers. These enzymes are reported to be environmentally friendly as compared to the conventional method. It was realized several decades ago that microbial enzymes might be useful in the processing of papers since it is composed of natural polymers such as cellulose, hemicelluloses, and lignin. However, microbial enzymes been commercially used in pulp and paper only in the previous decade while microorganisms are presently used in other industrial processing steps. This is due to the fact that wood and pulps which act as substrates are difficult to degrade. In addition, most research now focuses on lignin biodegradation since it is lignin that is removed from wood in chemical pulping and pulp in bleaching. Lignin likely evolved in part as a deterrent to microbial degradation, and it continues to be art impediment to biotechnological processing of wood and pulps. During the last decade, a rapid increase in the number of possible applications of enzymes in paper and pulp industries in which many are of commercial quantity has grown rapidly. These include xylanase for enzymatic bleaching, lipase for pitch removal as well as cellulase and hemicellulase for freshness enhancement (Kirk *et al*. 1996).

Globally our lives are somewhere governed by little inscriptions laid on paper in one way or the other. The major domains of the public sector are reliant on paper and paper products directly or indirectly. The history of paper dates back almost 2000 years, while the manufacturing process happened to be reported in China when inventors imprint their writings on crafted cloth sheets. The knowledge of papermaking then became popularized through westward and eventually reached India in around 605AD. Microbial enzymes applications in pulp and paper industries is rapidly increasing and thus reducing the adverse effects on the ecosystem. The enzyme also lead to the reduction of energy consumption, time and quantity of chemicals used in conventional deinking (Tavareset *et al*., 2014). During the deinking process, different steps which include washing, flotation, cleaning and screening are conducted in order to separate ink and other contaminants from waste paper **(Fig. 1.3)**. This usually depends on the type of waste paper and final deinked pulp. Removal of most types of ink and contaminants from

waste paper can be effectively conducted by either flotation singly or in combination with other processes (Jiang and Ma 2000). It is difficult to deink laser printed office papers by employing the conventional method of deinking. Due to the higher demand for laser printers and copy machines every year, there has been an increase in the amount of non-impact printed papers entering the recycled papers. It is challenging for the inventors to remove ink from these papers. The reason is primarily due to the strong adherence of the toner particles to the paper surface. The photocopier printers are indulged in using thermosetting toners made up of synthetic polymers as ink. Due to applying a high amount of heat during inking, the ink particles become physically bonded to the paper making it difficult as well as expensive to remove by a conventional method.

Waste paper recycling is another way that can preserve green plants and environment thereby preventing environmental pollution, water and energy (Singh *et al.,* 2012). The present conventional deinking method uses a large quantity of chemicals such (sodium hydroxide, sodium silicate, sodium carbonate and hydrogen peroxide), chelating agents and surfactants capable of damaging the environment (Zhang *et al.,* 2008; Pathak *et al.,* 2010). These chemicals when used for deinking may lead to the production of toxic effluents thereby increasing the amount of chemical oxygen demand (COD) in water and thus result in expansive wastewater treatment (Zhang *et al*., 2008). The discovery on the use of enzyme for the deinking process was found to solve most of the problems associated with chemical deinking methods since it is environmentally friendly. Microbial enzymes such as cellulase, xylanase, hemicellulase and pectinase are among the best enzyme produced by fungi and bacteria capable of altering the fibre surface to separate the ink which. Washing and flotation are used to remove the ink from pulp with aid of surfactants (Kirk *et al*., 1996; Xu *et al*., 2011; Gübitz, *et al*., 1998). The whole system of enzymatic deinking is an environmental friendly process; the enzyme production through Solid state fermentation (SSF) or submerge fermentation system help to reduce the bulk solid agricultural waste disposal and the enzymatic deinking process safe (Pathak *et al*., 2010).

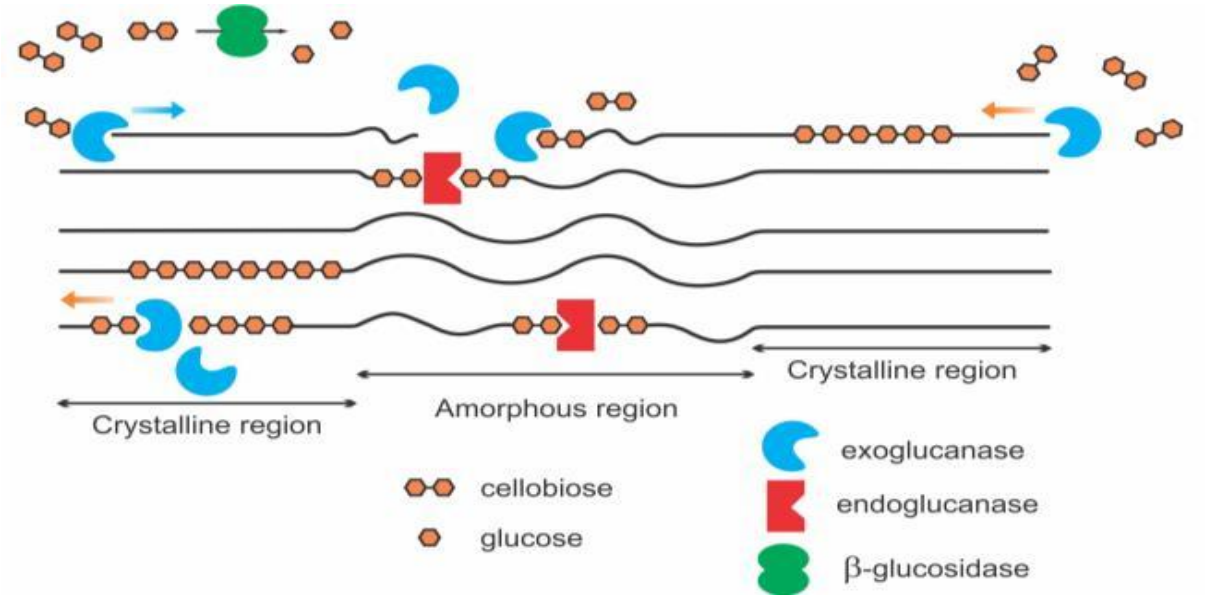


Figure 1.2: A simplified schematic representation of the enzymatic action of cellulase, involving exoglucanase, endoglucanase and β-glucosidase, on cellulose.

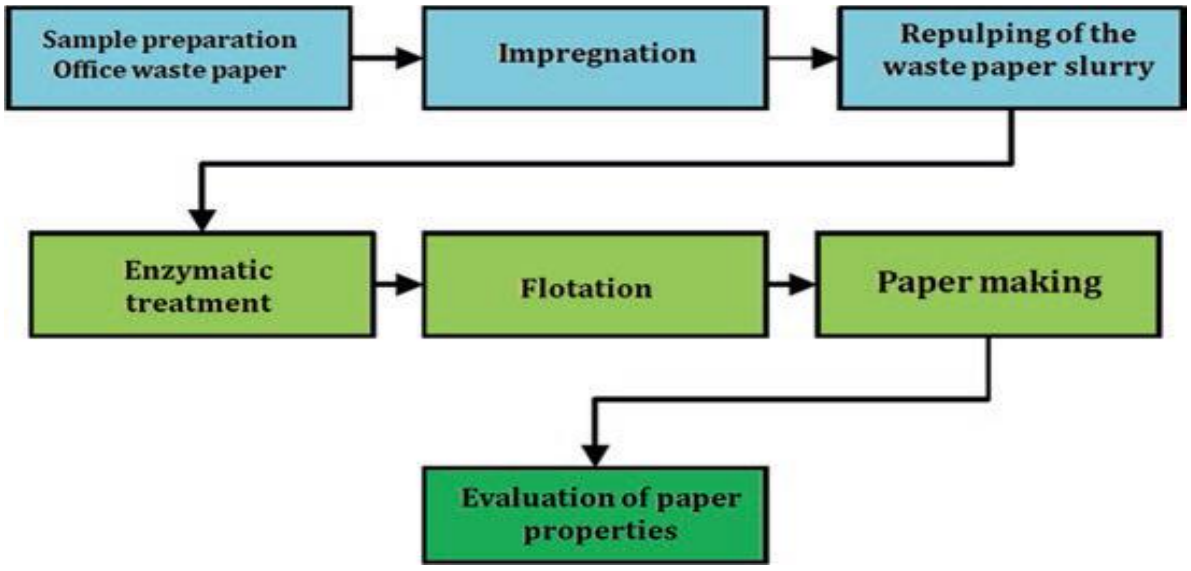


Fig: 1.3: Stages of Enzymatic Deinking of Wastepaper

In the last few decades, many papers reported production of microbial cellulase enzymes and bioconversion of cellulose, especially for paper industries. Presently, fermentation condition and cost of enzyme production are two main important factors of enzyme based bioconversion technology. Most of these researches usually reported the application of commercial cellulase enzymes for deinking purpose and found to yield a considerable improvement as compared to the conventional deinking method (Tsatsis, *et al.,* 2017; Zhang *et al*., 2008). Production of cellulase enzyme through fungi was also reported mostly within the range of acidic to neutral pH which is favorable for the growth for fungal growth (Roushdy, 2015; Pathak *et al.,* 2014; Soni, *et al.,* 2008). Although few research were conducted on alkaline fungal cellulase (Vega *et al*., 2012; Prasad *et al*., 2013; Muhammad *et al.,* 2011; Dutta *et al.,* 2018), non of such was carried out in Punjab region despite the fact that the soil of Kapurthala district is alkaline in nature and the state has abundant lignocellulosic waste.

Therefore, such research is needed in order to isolate, screen and identify potential alkaliphilic fungi that can be used to produce alkaline cellulase enzymes at low cost with a high activity using abundant lignocellulosic waste. These enzyme productions may be based on some factors such as temperature, pH, incubation time, inoculum size, carbon and nitrogen source. China, India, South Korea and Taiwan have recently emerged as industrialized manufacturing centre with strong national research and development programs, and play a great role in the world market of industrial cellulase production (Kikani *et al*., 2010). Looking at the fact that India is one of the largest paper producing countries with a large number of waste papers, several efforts were made to develop methods of deinking used papers so as to prevent frequent cutting down of trees which may cause deforestation and subsequently lead to soil erosion. However, since the soil of Kapurthala district in Punjab was found to be in alkaline condition, the research will focus on isolating, screening and identifying prospective alkaliphilic fungi capable of producing alkaline cellulase enzymes for cost-effective deinking waste paper since the conventional use of a chemical is very expensive and hazardous.

CHAPTER TWO

2.1 Review of Literature

Production of cellulase enzyme in particular and microbial enzymes in general, has been investigated considerably from a vast number of microorganisms both for research and industrial purposes **(Table 2.1)**. The production of cellulase from a fungal point of view was conducted by many researchers in a search to find their potential applications in different industries such as pulp, paper, textile, food, pharmaceuticals and biofuel. Ramanathan, *et al*., (2010) isolated *Fusarium oxysporum* from infected tomato plant parts for cellulase enzyme production using carboxylmethylcellulose as a source of carbon. Another *Fusarium* sp was studied under shaking conduction for the production of extracellular cellulase where β-glucosidase was produced on the first day of incubation (Trivedi and Rao, 1981)**.** From desert soil, Ahmed *et al*., (2018) isolated and screened *Fusarium dimerum* and *Rhizopus oryzae* strains capable of degrading cellulose using CMC as a carbon source. The exploitation of *Fusarium* sp in commercial quantity for cellulase production, Dutta *et al*., (2018) isolated, screened and identified three *F. solani*, three *F. oxysporum* and one *F. chlamydosporum* based on rDNA sequence analysis and their cellulolytic activities using CMC as carbon source. *Aspergillus hortai* with the potential of producing endoglucanase was also investigated under liquid state fermentation by El-hadi *et al*., (2014). In order to maximize cellulase production, nutritional and cultural parameters affecting CMCase production were optimized. Another fungal isolates capable of producing cellulase which includes *Aspergillus niger, Fusarium oxysporum, Fusarium avenaceum* and *Cephalosporium acremonium* were used for cellulase production using cellulose powder as a carbon source (Azzaz *et al*., 2012). In related work, Remaz *et al*., (2018) isolated, screened and identified three fungi as *Aspergillus niger, Fusarium solani* and *Trichoderma viride.* These were found to possess an ability to degrade cellulolytic materials from their natural environment of soil, tomatoes and oranges samples in Khartoum and Khartoum North regions of Sudan. Sasi *et al.,* (2012) isolated an *Aspergillus flavus* from a water sample of Tamil Nadu, India in a

search to find an alternative to chemical fungicides. Culture of these fungi produced cellulase enzyme under solid state fermentation using rice bran as the carbon source. Two methods were used for exoglucanase (FPase) enzyme production which includes incubating the enzyme with serially diluted leaf extract followed by enzyme assay or incubating the substrate (filter paper) with different concentration of leaf extract followed by enzyme addition and cellulase assay. From both methods, *F. oxysporum* was found with the highest percentage reduction activity than *N. crassa*. This is an indicated that cellulase enzyme produced from *F. oxysporum* in combination with aqueous extract of narrow–leaved peacock ginger can be used as a potential botanical fungicide to prevent plant infected with this fungus by reducing the cellulolytic damage of different plant parts (Basak and Rangan, 2018).

Cellulase in combination with other enzymes was also reported in some papers to improve productivity. Under solid state fermentation, cellulolytic and xylanolytic enzymes were produced from *Fusarium oxysporum* on corn stover and enhanced by optimization of nutritional and physicochemical parameters (Panagiotou *et al*., 2003). Xylanase was also produced from *Trichoderma viride*-IR05 using solid substrate fermentation. Sugarcane bagasse was found as the best carbon source among different lignocellulolytic waste examined. supplementation of xylose, tryptone, $NaNO_3$ and tween 80 as additional carbon source, nitrogen and surfactant respectively are found to improved xylanase (Irfan *et al.*, 2014).

2.2 Bacterial Strains Isolated from the Alkaliphilic Environment

The ability of microorganisms to breakdown cellulose is widely distributed among different bacteria and fungi. Among bacteria, a considerable number of Eubacteria like aerobic order *Actinomycetales* and anaerobic order *Clostridiales* possess such cellulolytic ability to degrade cellulose. George *et al*., (2001) isolated a novel *alkalothermophilic actinomycete* called *Thermomonospora* with optimum growth at pH 9 and 50^{O}C from self-heating compost. The organism was able to produce high amount of carboxymethyl cellulase (CMCase) enzyme purified under fractional ammonium sulphate precipitation followed by cellulose affinity chromatography and sepharcryl S-200 gel

filtration. While investigating and exploring possible sources of novel thermophilic species in natural products, a novel thermophilic and alkaliphilic actinomycete capable of producing alkaline cellulase from the soil of a tropical rain forest in Yunnan province China was isolated and identified (Wu *et al*., 2017). This strain named *Streptomyces thermoalkaliphilus* represents a novel species in the genus Streptomyces based on its phenotypic, chemotaxonomic and phylogenetic characteristics.

Kalpana and Rajeswari (2015) also reported *Streptomyces* from agricultural waste capable of producing enzymes for degrading xylan. *Streptomyces* sp are vital source of an enzyme involved in lignocellulosic degradation. The crude enzyme was found to have an application in deinking of newsprint. From sediment and water sample of alkaline Soda lake Maharastra India, bacteria such as *haloalkaliphilic Marinobacter excellens, Alkalimonas delamerensis, Roseinatronobacter monicus* and *Rhodobaca bogoriensis* were identified for the first time in Lonar lake (Borgave *et al.*, 2012). The bacterium described as *Heleococcum alkalinum* sp. novel was isolated on alkaline agar with carboxymethyl cellulose (CMC) and was a dominant species in samples of soda soils with pH >10 and relatively high salinity. This cellulolytic activity of an alkaliphilic obligate anaerobic bacterium, which was isolated from the microbial community of soda-lake sediments belonging to the cluster III of *Clostridia* with low G+C content was investigated by Zvereva *et al.*, (2006). The bacterium has the ability to grow in media with cellulose or cellobiose as the sole energy sources.

Table 2.1: Cellulase enzyme produced by different microorganisms

S/no.	Isolate	Enzyme	Maximum Enzyme produced (U/mL)	Reference
1	*Fusarium oxysporum*	Cellulase	(CMCase 1.92; FPase 1.34 and β-glucosidase 1.78)	(Ramanathan *et al*., 2010)
2	*Bacillus pumilus* 313SI	CMCase	3.08	(Goyal *et al*., 2014)
3	*Mucor indicus, M. hiemalis and Rhizopus oryzae*	Cellulase	86.0 U/gds for *Mucor indicus*, 9.0 *M. hiemalis* and 152.0 U/gds for *R. oryzae*	(Behnam *et al*., 2019)
4	*F. solani, F. oxysporum and F.chlamydosporum*	CMCase and FPase	CMCase (0.445) from *F. oxysporum* SF0801 FPase (9.25) from *F. oxysporum* SF1905	(Dutta *et al*., 2018)
5	*Trichoderma viride-IR05*	Xylanase	72.4±1.42U/g	(Irfan *et al*., 2014)
6	*Aspergillus hortai*	Endoglucanase	0.23	(El-hadi *et al*., 2014)
7	*Aspergillus niger*	Cellulase	0.097	(Azzaz *et al*., 2012)
8	*Aspergillus niger, Fusarium solani* and *Trichoderma viride*	Cellulase	2.90 from *Aspergillus niger*	(Remaz, 2018)
9	by *Aspergillus niger* BCC14405	endo-xylanase	59.7	(Khonzue *et al*., 2011)
10	*As. Flavus*	Cellulase	0.128	(Sasi *et al*., 2012)
11	*F. oxysporum*	endoglucanase, cellobiohydrolase, b-glucosidase, xylanase and b-xylosidase	xylanase (1840), β-xylosidaze (0.041), CMCase (304), cellobiohydrolase (4.1) and β-glucosidase (0.140U/g)	(Panagiotou *et al*., 2003)
12	*Bacillus pumilus* 313SI	CMCase	3.08	(Goyal *et al*., 2014)
13	*Bacillus sp.*	Amylase	604.17	(Zambare 2011)
14	*Enterococcus pseudoavium*	Amylase	6.05	(Sakthivel, *et al*., 2010)
15	Marine *Bacillus* VITRKHB	Cellulase	7.80	(Singh *et al*., 2014)

In various mangrove sites from Philippines, conventional as well as analytical profile index (API) were used to characterize and phenotypically identify five promising species of *Bacillus* producing cellulase enzyme and additional knowledge regarding the bacterial diversity of mangrove forests in the Philippines (Tabao and Monsalud 2014). An old newspaper (ONP) waste was described as a carbon source for growing *Bacillus subtilis* where avicelase and carboxymethylcellulase (CMCase) enzymes are estimated in the culture filtrate. *Bacillus subtilis* CMCase has more activity at optimal temperature and pH than Avicelase. Deinking with these enzymes brings about an increase in brightness of the sheet effective removal of ink particles and also prevents redeposition onto the fibre surfaces. These findings indicate that enzymatic deinking can perform better than the conventional chemical method Makky and Abdel-Ghany (2009). Another bacterium called *Bacillus halodurans* was purified to homogeneity and produce an extracellular haloalkaline cellulase by bioconversion of lignocellulosic waste by (Annamalai *et al.*, 2013). This indicates that purified cellulase produce from *Bacillus halodurans* utilizing lignocellulosic biomass could be of great potentials in industrial process. Other enzymes such as xylanase can be produced by alkaline bacteria. *Bacillus pumilus* ASH was found to have the ability to produce an extracellular and thermostable xylanase enzyme. The enzyme activity was slightly lower in solid state fermentation (SSF) than in submerged fermentation technique but the ability of the organism to produce such a high level of xylanase at room temperature, with deionized water and with no addition of any mineral salts in SSF could lead to a substantial reduction in the overall cost of enzyme production. This is the first time a high level of xylanase was on produced under SSF conditions by bacteria (Bindu *et al*., 2006). Similar bacterium *Bacillus pumilus* SV-205 was also reported to produce xylanase (Nagar *et al*., 2012). Other bacteria producing different enzymes include *Enterococcus pseudoavium* which produce amylase enzyme (Sakthivel *et al*., 2010) and *Bacillus* sp which also produce amylase (Zambare 2011).

2.3 Fungal strains Isolated from the Alkaliphilic Environment

Just like bacterial enzymes, alkaline cellulase enzymes produced from alkaliphilic fungi are reported to have huge biotechnological applications in many industrial settings such as textile, paper, food, detergent and biofuel. However, their industrial applications have been hindered due to the lack of microbial strains that can produce a considerable amount due to high pH. Over the past few decades, researches on alkaline cellulase have generated attention on cellulase production at alkaline pH. In search of alkaline cellulase enzyme, Kang and Rhee (1995) reported an alkaline *Cephalosporium* sp RYM202 while desert *Basidiomycetes* strain of unknown origin was reported by Sreenath *et al*., (1996). Two extracellular alkalistable endoglucanase from alkalotolerent *Fusarium* sp was reported by Vyas and Lachke (2003). The enzyme can increase pulp brightness with a reduction in ink count of recycled waste paper. With more interest in the production of alkaline cellulase enzyme, a considerable amount of alkaline cellulase was produced from extremophilic filamentous *Penicillium citrinum* with potential effectiveness as additives to laundry detergent (Dutta *et al*., 2008). In addition, alkaline cellulase was from alkalotolerent *Chaetomium* sp isolated from mangrove leaves using agricultural and industrial wastes as substrate (Ravindran *et al.,* 2010) while Hmad *et al*., (2014) produced alkaline cellulase from *Stachybotrys microspora*. In another research by Kladwang *et al*., (2003), about 490 alkaline tolerant fungi from a natural environment using Petri dishes containing potato dextrose agar medium buffered at pH 11.0 were identified from different habitats in Thailand. This research indicated that a good source of alkaline enzyme production can be found from alkaline tolerant fungi isolated from tree holes in alkaline environments.

Soil is one of the favorable niches for isolation of alkaline microorganisms. In Peru, soil from an undisturbed forest was investigated for fungi capable of producing alkaline cellulase. The best producers of cellulase in highest productivities are the *Penicillium sp* LM-HP33, *Penicillium sp.* LM-HP37 as well as *Aspergillius sp* LM-HP32. These fungal strains are found to be suitable for the production of alkaline cellulase

(Vega *et al*., 2012). High cellulase activity was found from a fungal strain in subtropical soils having a medium supplemented with rice straw by Picart *et al*., (2007). Crude cellulase produced by *Penicillium sp*. CR-316 has potential in industrial applications since it showed activity and stability at high temperature and produce a thermostable cellulase. Bilanenko *et al*., (2005) reported an isolate representing a group Ascomycete from saline soda soils of Central Asia and Africa.

2.4 Optimization of Microbial Enzymes using a Statistical Approach

Composition medium and fermentation conditions were reported to influence production and optimization of cellulase by microorganisms. Physical parameters like pH, temperature, and incubation time as well as nutritional factors like carbon and nitrogen sources were found to be the major factors affecting cellulase production (Hao and Hung, 2013). Several efforts were made for high production of cellulase by optimization of best possible fermentation conditions. One factor at a time (OFAT) approach which time consuming and very expensive is one of the classical methods usually applied for this process. A combination of interactions between physical and nutritional parameters for the production of cellulase is numerous and an optimum process can be designed using an effective statistical and experimental design procedure. A collection of statistical techniques called Response Surface Methodology (RSM) can be used for designing experiments, models as well as evaluating the effects of parameters for optimum production has been implemented for optimization of cellulase enzymes (Singh and Kaur 2012). Contrary to conventional techniques, statistical tools like SRM have gained considerable attention for the production of different microorganisms due to its application in many industrial processes. Aanchal *et al*., (2016) reported a 20-fold increase in cellulase using second-order central composite design (CCD) of an experiment in response surface methodology. Different nutritional parameters such as wheat bran, magnesium sulphate and calcium chloride concentration as well as physical parameters such as pH and temperature were optimized using response surface methodology for the production of cellulase using *Schizophylum commune* NAIMCC-F-

03379 isolated from decomposed leaf sample of *Lantana camera.* Under optimizes condition, 5.35-fold increase and 6.62-fold increase were reported for CMCase and FPase respectively (Kumar *et al*., 2018).

This powerful and effective mathematical optimization approach was also used for the production of cellulase from *Trichoderma reesei* RutC30 using agricultural waste (rice straw and banana fibre) as the source of carbon through submerged fermentation as reported by (Muthuvelayudham and Viruthagiri, 2010). They identified temperature, pH, substrate concentration, inducer concentration, inoculum age and agitation speed as the most important optimizing parameters for the production of cellulase. Same *Trichoderma reesi* RUTC30 was also used for the optimization of cellulase production using sugarcane bagasse as a carbon source by Mekala *et al*., (2008). The research was reported to optimized parameters such as temperature, incubation time and inducer concentration using Box-Behnken experimental design. The result indicated the highest FPase production of 25.6U/gds when inducer concentration was 0.33ml/gds, temperature of 33^{O}C and incubation time of 67hrs. Saravanan *et al*., (2012) reported an increase of cellulase production of 9.23U/ml and 6.98U/ml from BBD and genetic algorithm (GA) respectively when *Trichoderma reesi* was used for enzyme production by optimizing fermentation condition parameters such as pH, temperature, initial substrate concentration, inoculums concentration and incubation time. The result showed that BBD statistical tool gave more enzymes as compared to genetic algorithm.

A study was conducted to optimized cellulase production for efficient dinking and enzymatic hydrolysis of solka floc and bagasse from a versatile *Aspergillus fumigatus fresenius* (AMA) by Box-Behnken Design (BBD) of an experiment from RSM. The CMCase was capable of removing 53% residual ink and increase brightness of the hand sheet by 4.32% ISO (Soni *et al*, 2010). In related work, nutritional and fermentation conditions were optimized for the production of cellulase and pectinase from *Aspergillus oryzae* using solid state fermentation. Response surface methodology (RSM) as a statistical tool was used through BBD to optimized moisture content, pH, temperature and incubation time which produced a maximum cellulase and pectinase that lead to a

significant increase in enzyme productions as compared to conventional one factor at a time approach (Hao and Hung, 2013). Conditions for the production of CMCase from *Aspergillus nidulans* SU04 and *Aspergillus nidulans* MTCC344 under solid state fermentation were optimized using central composite design (CCD) (Jabasingh, 2011). A newly isolated *Aspergillus niger* HN-1 was also used for the production and optimization of cellulase by Plackett-Burman and central composite design statistical models. The design expert software was capable of improving FPase and β-glucosidase activities by 2 and 3 fold increases respectively (Sandhu *et al*., 2013). Three filamentous fungi which include *Mucor indicus, M. hiemalis* and *Rhizopus oryzae* were produced from solid state fermentation using wheat bran as sole carbon source. RSM was used to optimize fermentation parameters which include temperature, incubation period and moisture content for the production of cellulase (Behman, *et al*., 2019).

A considerable number of bacteria were also found to produce a high quantity of enzymes when optimized for potential industrial applications through response surface methodology. Singh and Kaur (2012) isolated 30 potent bacterial species as potentials cellulase producers of which isolate JS 14 produced the highest cellulase. The isolate was later identified as Bacillus sp based on morphological and biochemical reactions. A cellulase enzyme produced by *Bacillus pumilus* EWBCM1 was optimized to produce a maximum cellulase when parameters such as galactose, malt extract and incubation time were optimized using RSM based on the Central Composite Design (Singh *et al*., 2014). This statistical software was used for the optimization of other enzymes of industrial applications produced from different microorganisms. Lipase isolated from *Aspergillus niger* strain AC-54 was optimized initially through Plackett-Burman (PB) design followed CCD. The predicted activity of this enzyme from the statistical model was validated and confirmed by the experimental result (Contesini *et al*., 2009). Form a slaughterhouse polluted with water, a keratinolytic enzyme producing bacterium was isolated and identified as *Bacillus pumilus* A1. Placket-Burman was initially applied to identify the best ingredients and conditions for maximum production of keratinase. Optimization of five important parameters which includes feather meal, soy peptone,

NaCl, KCl and KH_2PO_4 were conducted by central composite design where a 3.4-fold increase keratinase production was generated as compared to Placket-Burman (Fakhfakh-zouari *et al*., 2010). In similar research, a full factorial of the central composite design was applied in evaluating the effects of major amylase production parameters isolated from *Bacillus* sp under submerged fermentation (Zambare, 2011). A potential application of crude endo xylanase for biobleaching of chemicals from eucalyptus produced by *Aspergillus niger* BCC14405 was also investigated by Khonzue *et al*., (2011). Response surface methodology as a statistical tool was used for the optimization of fermentation parameters. The optimized enzyme was able to increase brightness and pulp viscosity suggesting an increase in cellulose content.

2.5.0 Waste Paper Deinking Process

The chemical deinking process mainly employed by pulp and paper industries for recycling purpose has been reported to cause environmental effects. Majority of these chemicals like sodium hydroxide and sodium silicate are fairly standard while others are said to be complex with many functions (Pala *et al.*, 2004). The chemicals are used to separate ink and other contaminants from the fibre. Based on the cost implication as well as strict environmental regulations, there is a need for paper industries to embark on a better recycling technology that can be environmentally friendly (Jiang and Ma 2000). Over the past decades, many papers are reported to have successfully used enzymes as a replacement to conventional chemical deinking (Pala *et al*., 2004; Pathak *et al*., 2014; Poorna and Prema, 2007). In a related development, Lee *et al.*, (2011) reported an improvement of brightness, tensile index, burst index, freshness and residual ink concentration from enzyme deinking as compared to the conventional method. However, some paper suggested an improvement of paper quality when the chemical was combined in an equal profession with the enzyme. A commercial cellulase enzyme was found to have less efficient deinking when compared to either alkaline or sulphite chemistry. However, a combination of the said cellulase enzyme with sulphite showed a promising improvement of ink removal (Zhang *et al*., 2008)**.**

A biological process using enzymes had been evaluated which implied positive results in the deinking process of different types of waste paper. One of the major advantages of using enzymes is the production of minimum treatment effluent and also less harmful to the environment. The most important step involved in the recycling process is the removal of ink from the paper. Several microbial enzymes either individually or in combination are found to be applicable in the removal of ink from waste paper. Several enzymes like cellulase, hemicellulase, α-amylase, lipase, xylanase and other lignolytic enzymes are involved in the biological process of deinking **(Table 2.2)**. The enzymatic treatment is favorable because enzymes are eco-friendly in nature and during its processing ink detachment occurs without any discharge of harmful chemicals, thus rendering our environment green. Most of the cited studies reported deinking of mixed office waste consisting of photocopier paper using a commercially available enzyme (Roushdy 2015). India generates approximately 36.5 million tones of municipal solid waste annually, of which 14.6 million tones consist of paper wastes. The Indian Agro and Recycled Paper Mills Association (IARPMA) estimated that India is among countries with low recycling of waste papers (26%) as compared to countries like China (38%), Thailand (45%) and Germany (80%). Based on the shortage of raw materials, resources and high demands being imposed on green plants, Indian paper industries are facing many challenges on daily basis. One of the attractive solutions to these problems in India is the recycling of municipal office waste (MOW) papers but still very difficult to remove non-impact ink.

Deinking process which involves the removal of printed ink from used paper involves dislodgement of ink particles from fibre surface and the separation of dispersed ink from the fibre suspensions by washing and flotation (Tavares *et al*., 2014). In the chemical deinking process, industries used a large quantity of chemicals such as chlorine, chlorine based derivatives, sodium hydroxide, sodium carbonate, sodium silicate, hydrogen peroxide, hypochlorite, and chelating agents, which lead to hazardous effluent disposal problems (Vega *et al*., 2012; Pala *et al*., 2004). For these reasons, biological deinking by using microbial enzymes which act directly either on the fibre or on the ink

film becomes more attractive. For example, hydrolysis of cellulose and hemicellulose brings detachment at fibre/ink inter bonding regions and finally release ink particles into the suspension when treated with cellulase/hemicellulase enzymes (Lee *et al*., 2007). Same microbial enzymes can also detach small fibrils from the surface of the ink particles hence changes the usual hydrophobicity of the particles, which brings about their separation in the flotation/ washing step. This enzymatic technology has been described as especially advantageous in deinking high-quality waste papers, whose reuse is usually limited as the non-impact inks (toners) polymerize onto the paper surface using thermoplastic binders during the high-temperature printing process. In the chemical deinking process, the toner particles usually remain large, flat and rigid, and separate very poorly from papers during the fibre/ink separation stages (Jiang and Ma, 2000).

2.6. Cellulase and its Applications in Paper Industry

Microbial cellulases have been focused as one of the important biocatalysts being multiplex in nature and bearing extensive applications. Cellulase and hemicellulase enzymes are both synthesized by fungi and bacteria (**Table 2.3)**. As compared to fungi, bacteria have a high rate of cellulase enzyme production rate due to its advantage of high bacterial growth rate. The most important parameters for a successful production of cellulase enzyme are the screening of the organism, optimization of fermentation conditions and selection of substrate. Using carboxymethylcellulose as substrate, (Ariffin *et al*., 2006) produced enzyme cellulase from the local isolate of *Bacillus pumilus* EB3. This enzyme screened from this bacterium was purified using ion exchange chromatography using anion exchanger for cellulase characterization.(Rawat and Tewari 2012) isolate and identify microorganism which hydrolyzed carboxymethylcellulose (CMC) as *Bacillus subtilis* strain LFS3. Gel filtration chromatography, ion exchange and sodium sulphate precipitation are the methods used to isolate and screen enzyme cellulase with an overall recovery of 15%. Optimum temperature and pH for an active profile of this enzyme were $60^{O}C$ and 4.0 respectively.

Table 2.2: Microbial enzyme and their applications in pulp and paper industry

S/no.	Microorganism	Enzymes	Application	Ref.
1	Commercial enzymes	Cellulase	Ink removal, freshness and reduction of drainage time	(Pathak *et al*., 2011)
2	*Trichoderma, harzianum*	Cellulase and xylanase	Improved drainage, high deinking efficiency, brightness and reduce drainage time	(Pathak *et al*., 2014)
3	Commercial enzymes	Cellulase	Not good if specks surface of the deink paper are use	(Tsatsis *et al*., 2017)
4	Commercial enzymes	Cellulase	Detach significant amount of ink from ONP/OMG	(Zhang *et al*., 2008)
5	*Aspergillus niger*	Cellulase and hemicellulase	Enhanced optimum deinking efficiency	(Lee *et al*., 2007)
6	*Streptomyces sp.* L22001	Xylanase	Biobleaching effect	(Li *et al*., 2010)
7	*Bacillus altitudinis*	Xylanase	Potential for biodeinking and biobleaching	(Adhyaru *et al*., 2017)
8	*Bacillus sp.* CKBxID	Xylanase	Deinking agent of recycled waste paper	(Maity *et al*., 2012)
9	*Alkalothermotolerant*	Xylanase and pectinase	Commercially viable with better paper quality	(Singh *et al*., 2012)
10	*Aspergillus niger*	Xylanase	Deink old newspaper with improved brightness, removal of surface ink particles from ONP pulp	(Desai and Iyer 2016)
11	*Aspergillus nidulans*	Xylanase	Reduction of ink and increased brightness of recycled paper	(Taneja *et al*., 2002)
12	Commercial enzymes	Laccase	Reduction of lignin content and useful in the process of biopulping	(Nagar and Nagar 2014)
13	*Enterococcus pseudoviun*	Amylase	Effectively deink and decolorize paper pulp within four days incubation	(Sakthivel *et al*., 2010)
14	Commercial enzymes	Laccase and hemicellulase	Deink old newspaper	(Xu *et al*., 2011)
15	Commercial enzymes	Cutinase and amylase	Increase brightness and ink removal	(Wang *et al*., 2018)
16	Commercial enzymes	Amylase and cellulose	Improve brightness	(Gil *et al*., 2013)

A fungus called *Coprinopsis cinerea* was found to have the ability to produce a crude cellulase and xylanase enzymes with potentials of deinking photocopier waste paper deink photocopier waste papers as reported by (Pathak *et al*., 2014). In their view to achieving maximum and possible efficiency without affecting paper and its strength properties, enzyme dose, point of enzyme addition, pulp consistency and reaction time were investigated which also confirmed the potential of a crude enzyme of *C. cinerea* for deinking of photocopier waste papers

Effects on the use of cellulase for deinking of office waste paper was investigated by (Tsatsis *et al*., 2017). Better results were achieved by the use of enzyme in the deinking experiment as compared to those in which enzymes are inactive. It was discovered that enzyme application has a disadvantage if specks surface of the deink paper sheets was uses as compared conventional deinking. Based on their finding, more research is needed in formulations of an enzyme with better performance under alkaline conditions as well as the types of paper printed in the different photocopier and laser printers. (Abo-State *et al*., 2013) isolate *Bacillus* strains from agricultural waste and identified as *Bacillus thuringenesis* which have the ability to produce cellulase and xylanase based on their pH and temperature. Stability at different temperatures (60-80°C) at separate duration was also investigated**.** Zhang *et al*., (2008) evaluate three commercial cellulase enzymes for their application on deinking artificially aged old newspaper (ONP) mixed with fresh old magazine (OMG) in a ratio of 7:3. At the start of repulping, these enzymes were added followed by incubation for 3hrs. Despite the fact that cellulase enzyme was able to remove a significant amount of ink from ONP/PMG, they have low efficiency than using conventional methods of either sulphite or alkaline deinking chemistry. None of the three cellulase enzymes tested were able to separately deink aged ONP/OMG, and a poor deinkability was also observed by using either sulphite or alkaline chemistry. However, the research indicates a significant increase of deinking when a combination enzyme and sulphite are applied which provide a potential strategy of achieving effective deinking of old newspapers at neutral pH. Similarly, Rohit *et al*., (2008) reported *Aspergillus sp.* AMA, *Aspergillus terrus* ANI and *Myceliopthora fergusii*

T41 from composting soil capable of producing different enzymes for their deinking capabilities. These fungi have shown a significant deinking capability on composite waste paper. They were found to produce multiple endoglucanases and are characterized to lack a cellulose-binding module (CBM) which can be attributed for their better deinking capability.

Enzymatic deinking has added advantages over conventional deinking as it reduced alkali usage, improving fibre brightness, and greater strength property. Moreover, an enzymatic deinking process also prevents alkaline yellowing and reduces environmental pollution. In an effort to increase the rate of production, fungal cellulase has been pursued by several mills in improving the drainage. These enzymes are also used in the production of easily biodegradable stationary objects including paper towels and sanitary paper (Kuhad *et al*., 2011). Laccase mediator system was used in a study conducted to identify the similarity on the application of cellulase/hemicellulase for deinking printed fibres from newspapers and magazines. In this regards, commercially available endoglucanase and endoxylanase activities and a commercial laccase were evaluated in the presence of synthetic or natural mediators. They concluded that factors to be considered for the application of enzymatic deinking processes in addition to enzymes includes ink types, printing methods, and fibre/ink separation process (Ibarra *et al*., 2012). Lee *et al.,* (2007) also developed a laboratory procedure for enzymatic deinking of waste papers using cellulase and hemicellulase enzymes produced from *Aspergillus niger*. Using an optimized floatation system at 6.0 pH and 45^{O}C temperature, an optimum deinking efficiency with these enzymes was enhanced to 95%. The deinked papers are found to have similar properties with commercial papers indicating the effectiveness of the developed enzymatic process.

Table 2.3 Cellulase enzymes and their optimum pH and Temperature produced by different microorganisms

Sample	Location	Organism	Enzyme	PH	Temp (^{O}C)	Ref.
Soil	Pulp and paper industries, India	*Bacillus subtiliss*	Cellulase	4.0	60	Pala *et al.*, (2004)
Soil	Macuya rain forest, Pucallpa, Peru	*Aspergillus sp* LM-HP32, *Penicelium* sp. LM-HP33 and 37	cellulase	4.8-9.4	28	Vega *et al.*, (2012)
Soil, compost, animal waste slurry	Jeju island, South Korea	*Bacillus subtilis* C5-16 and S52-2	CMCase, avicelase, xylanase	5.0	50	Kim *et al.*, (2012)
Wild herbivore, rain deer	Wayanad, Kerala, India	*Escherichia coli* SD5	Cellulase, xylanase	NA	37-39	Kumar *et al.*, (2018)
Agricultural waste	Cairo, Egypt.	*Bacillus thuringenesis* MAM-29, MAM-38	Cellulase and xylanase	3-7.6	60-80	Abo-State *et al.*, (2013)
Soil	Iguazo rainfalls, Argentina	*Penicillium sp* CR-313 and *Penicillium sp.* CR-316	Cellulase	4.5	65	Picart *et al.*, (2007)

2.7 Other Microbial Enzymes and their Application in Pulp and Paper Industries

A high amount of xylanase enzyme was produced from *Bacillus pumilus* SV-205 using optimized fermentation conditions. The bacterium secretes maximum amount of cellulase free xylanase in combination with yeast and peptone which also enhanced the highest xylanase production that differs from other combinations (Nagar *et al.*, 2012). Li *et al.*, (2010) produced xylanase with bio bleaching potentials on wheat straw pulp from *Streptomyces* L2001 with a stable optimum temperature of 70^{O}C and pH of 5.3. High production of xylanase from another bacterium called *Bacillus altitudinis* DHN8 followed by its purification and application was also reported by Adhyaru *et al.*, (2017). Using response surface technology, enzyme yield was improved by optimizing submerged fermentation conditions which lead to a two-fold increase in activity as compared to activity in one factor at a time optimization. The research indicates a potential use of *Bacillus altitudinis* for bio deinking and bio bleaching. For pollution-free environment, the recently employed technique is the recycling of civic paper waste by enzyme based technology. In these regards, a newly isolated *Bacillus sp* CKBx1D for recycling of laser jet paper waste was isolated for its potential ability to purify xylanase enzyme by Maity *et al.*, (2012). Response surface methodology was the technique used to optimize all operational parameters while the purified enzyme mixture was used for laser printed paper waste deinking potentials. The bacterial isolate and its xylanase enzymatic system could efficiently be used in recycling paper waste as a deinking agent. According to Gessesse and Mamo (1999), the overall cost of xylanase enzyme production from *Bacillus sp.* AR-009 can be greatly reduced using solid-state fermentation technique. The ability of the organism to produce high xylanase activity at alkaline pH and lower wheat bran to moisture ratio could have a potential advantage in minimizing the risk of contamination.

Production of xylanase from the fungal point of view was also reported in some papers. Dutta *et al.*, (2007) studied the culture filter of *Penicillium citrinum* grown on wheat bran bed in solid state fermentation to purify an extracellular xylanase enzyme.

Moderately thermostable xylanase showed optimum activity at 50^{O}C at pH 8.5. Purification and characterization of this novel endoglucanase free alkaliphilic xylanase from the alkali tolerant fungus *P. citrinum* were discovered for the first time which may have potential applications in paper and pulp industries. Desai and Iyer (2016) reported production of cellulase free xylanase enzyme from fungi for deinking of Old News Paper (ONP) pulp from *Aspergillus niger* strain DX-23. Other xylanase enzymes with reported application in pulp and paper industries includes thermostable xylanase produced from alkalophilic fungi called *Aspergillus nidulans* KK-99 (Taneja *et al.*, 2002) and xylano pectinolytic enzymes for deinking of school waste paper was conducted by Shatalov and Pereira (2008).

Other enzymes of potential application in pulp and paper includes were also reported. *Candida albidus* for example is responsible for producing laccase that helps in the reduction of lignin content found in the eucalyptus wood and happen to be useful in the process of bio pulping. It also efficiently removes toxic effluents coming from the pulp mills which contain a significant amount of phenolic compounds and Chlorolignins (Kaur and Nigam 2014). The ink removal capability of laccase and xylanase enzymes from an alkalophilic bacterium on recycled old newspaper (ONP) in combination with the physical deinking method of sonication and microwaving was reported (Virk *et al.*, 2013). An enzyme showing alkaliphilic laccase activity was purified from the culture supernatant of *Myrothecium verrucaria* 24G-4 by (Sulistyaningdyah *et al.*. 2004). The enzyme was very stable in alkaline conditions with an optimum pH of 9.0 and was able to remove synthetic dyes under the same conditions which confirm the function of this enzyme in an alkaline environment.

Amylase is one of the important enzymes produced by bacteria and fungi in large quantities which can be used commercially. *Aspergillus niger* has a significant amount of hydrolytic capacities in the production of α-amylase (Sahni and Goel 2015). Sakthivel *et al.*, (2010) isolated and screened some bacteria that inhabit decaying vegetables for the production of amylase enzyme. When cultured with paper pulp, the bacterial culture

immobilized in sodium alginate beads can decolorize this paper within four days. This shows the ability of extracellular amylase produced from *Enterococcus pseudoavium* to effectively deink and decolorize paper pulp within four days of incubation (Singh *et al.,* 2012). Both bacterial lipase and fungal lipase are widely used in different industries. Lipase producers include bacteria, fungi, yeast and actinomycetes. Examples of fungi producing lipase are *Acinetobacter radioresistens*, *Aeromonas hydrophila*, *Aspergillus oryzae* (Andualema and Gessesse 2012). A thermophilic *Bacillus coagulans* BTS-3 was reported to produce alkaline lipase. The bacterium can be enhanced substantially when parameters like nitrogen source, carbon source and initial pH of culture medium were consecutively optimized (Kumar *et al.,* 2005). For the first time, Wang *et al.,* (2018) reported the effect of cutinase enzyme on the deinking of mixed office waste paper (MOW). Combination of cutinase, amylase, and some complicated surfactants can be used to replace the conventional chemical deinking methods at the neutral deinking process. When these enzymes are treated in a combination of surfactants mixed with pulp and office waste paper at a temperature of 50^{O}C for 30mins, the brightness, ink removal rate, tensile index and tear index of the deink paper will increase significantly. Hemicellulase was combined with laccase mediator system (LMS) by Xu *et al.,* (2011) to deink old newsprint (ONP). The result indicates the effective residual ink concentration was lower as compared to pulp deinked with hemicellulase or LMS individually. According to this study, an environmentally friendly and effective deinking method can be achieved when cutinase and amylase enzymes combine with cardanol polyoxyethylene ether and other surfactants.

From the past studies, it has been concluded that most of the microorganisms capable of producing enzymes especially fungi are normally grown in acidic to neutral pH. However, few reported research on alkaline cellulase enzymes were not evaluated for application in pulp and paper industries. As previously reported the disadvantages of using conventional deinking methods by industries due to its cost, environmental effect hazardous chemicals as well as reported different commercially available enzymes as a replacement to chemical deinking, most of these enzymes are expensive. The available

method of cellulase enzyme production from bacteria and fungi is cheaper and eco-friendly. It also decreases the significant amount of chemicals which are indulged in order to get brightness in chemically bleaching process. In the conventional method of papermaking process, the manufacturers use hazardous chemicals which impart negative impact to the environment (Khonzue *et al.*, 2011). Moreover, looking at the physicochemical condition of a chemical deinking method with high pH and temperature, isolation of thermoalkaliphilic fungi that can produce a considerable amount of endoglucanase enzyme for deinking purpose will be of paramount importance. Therefore, there was focused on isolating novel thermoalkaliphilic fungi from Kapurthala district, Punjab from the soil and industrial effluents.

CHAPTER THREE

3.1 RESEARCH OBJECTIVES

1. Isolation and screening of alkaliphilic cellulase producing fungi from the soil and industrial effluents
2. Identification of fungal cultures based on microscopic and molecular characterization by 18s rRNA sequencing
3. Production and optimization of physic-chemical and nutritional parameters for alkaliphilic cellulase producing fungi using Response Surface Methodology (RSM).
4. Evaluation of cellulase enzymes for deinking potentials in the pulp and paper industry

CHAPTER FOUR

4.1 Hypothesis

The fast depletion of forest resources and its impact on the ecological balance has forced the paper industries to turn to the use of waste papers. The conventional recycling of waste papers uses chemical such as sodium hydroxide, sodium silicate, hydrogen peroxide as well as chelating agents and surfactants, which are not only costly but also hazardous to the environment. These chemicals when used for deinking may lead to the production of toxic effluents thereby increasing the amount of chemical oxygen demand (COD) in water and thus result in expansive wastewater treatment. The discovery on the use of enzymes for deinking process was found to solve most of the problems associated with chemical deinking methods since it is environmentally friendly. Microbial enzyme applications in pulp and paper industries are rapidly increasing and thus reducing the adverse effects on the ecosystem. The enzymes also lead to the reduction of energy consumption, time, and quantity of chemicals used in conventional deinking. In the last decades, research has been aimed at producing cellulase and other enzymes from fungi and bacteria for deinking of waste papers as a substitute to present conventional methods. However, most of these researches reported fungal endoglucanase production for recycling waste paper at acidic to neutral pH which is the best condition for fungal growth. Looking at the fact that industrial waste paper recycling usually occurs at relatively high pH and temperature, isolation, and screening of fungi capable of producing a high amount of cellulase enzyme at alkaline conditions become necessary. However, since the soil of kapurthala district in Punjab is naturally alkaline, the research will focus on isolating, screening, and identifying prospective alkaliphilic fungi capable of producing alkaline cellulase enzymes for cost-effective deinking waste paper since the conventional use of a chemical is very expensive and hazardous.

CHAPTER FIVE

5.0 Research Methodology

5.1 Sampling Sites and Sample Collection

Soils of different villages as well as industrial effluents from Wahid Sandhar Sugars Ltd Phagwara were selected from Kapurthala district and another site was industrial effluent of Khanna paper mills, Amritsar region of Punjab. Samples were collected as per the method discussed in Bairagi *et al.,* (2016). Soil samples were collected from eight different villages in Kapurthala district. These villages include Domeli, Dhoda, Ucha, Randhipura, Natpur, Fattudhinga, Fatehpur, and Samana. The samples were taken from the depth of 1-15 inches from the topsoil and sieve with 2mm wire mesh. The sieved soil samples were dispersed in sterile polythene bags and brought to the laboratory for analysis. The industrial effluents from Khanna paper mills Amritsar and decomposing bagasse from Wahid Sandhar Sugars Ltd Phagwara were also aseptically collected from sterile sample bottles and polythene bags respectively, kept at $4^{O}C$ for further laboratory analysis.

5.2 Isolation Fungal Cultures

Isolation of fungi was conducted according to a method of Ram *et al.,* (2014) as well as Salar and Aneja (2006). The fungi were isolated using serial dilution method in which 1g of soil was transferred into 10ml distilled water in a 100ml conical flask and stirred for complete homogenization. Dilutions were made up to 10^{-4} and 1ml of the serially diluted samples was poured on to sterile Petri plates. A Mandel and Reese media containing the following composition in g/L Protease peptone 1.0, Ammonium sulphate (NH_4SO_4) 1.4, Potassium dihydrogen phosphate (KH_2PO_4) 2.0, Urea (NH_2-CO-NH_2) 0.3, Magnesium sulphate ($MgSO_4.7H_2O$) 0.3, Calcium chloride ($CaCl_2$) 0.002, Ferrous sulphate ($FeSO_4.7H_2O$) 0.005, Manganese sulphate ($MnSO_4.H_2O$) 0.001, Zinc chloride ($ZnCl_2$) 0.017, Agar-Agar 15, Carboxy Methyl Cellulose (CMC) 10 and cellulose powder 10 were prepared and sterilized by autoclaving at $121^{O}C$ and 15 lbs pressure separately.

The pH of the medium was adjusted to different alkaline level (7-11) using 1N NaOH and 1N HCl. After autoclaving, 50mg/L of antibiotic amoxycillin was added to prevent bacterial growth and a 20 ml of this medium was transferred on to sterile Petri plates containing 1ml of the diluted soil sample and allowed for solidification and incubated at 30°C for 8 days. The fungal cultures grown on the medium were subcultured for repeated times to get a pure colony. The pure culture was transferred on to the Potato dextrose agar (PDA) slants and maintained at 4°C for further studies. Isolation of fungal culture from industrial effluent was prepared similarly as mentioned above.

5.3 Screening of Alkaline Cellulolytic Fungi (Congo Red Test)

Screening of fungal cultures were carried out as per the work of (Bairagi *et al.,* 2016) as well as Tabao and Monsalud (2014). The isolated fungal cultures were screened for their ability to produce cellulase enzymes using Mandel and Reese media. The broth media contained in (g/l) of the following composition:

Protease peptone	1.0
Ammonium sulphate $(NH_4)_2SO_4$	1.4
Potassium dihydrogen phosphate KH_2PO_4	2.0
Urea NH_2-CO-NH_2	0.3
Magnesium sulphate $MgSO_4.7H_2O$	0.3
Calcium chloride $CaCl_2$	0.002
Ferrous sulphate $FeSO_4.7H_2O$	0.005
Manganese sulphate $MnSO_4.H_2O$	0.001
Zinc chloride $ZnCl_2$	0.017
Agar-Agar	10

Carboxy Methyl Cellulose (CMC)	10
Distilled water	1000ml

Mandel and Reese medium supplemented with 1% CMC was used with pH medium adjusted to different alkaline range from 7 to 11 using 1N NaOH and 1N HCl. After autoclaving at 121°C and 15 lbs. pressure, 50mg/L of antibiotic amoxycillin was added to prevent bacterial growth. Liquid medium 100ml was dispensed in 250ml flask. The flasks were then inoculated with an actively growing fungal culture cut with cork borer of 1cm diameter. The flasks were incubated at 30^{O}C in a rotary shaker for eight days to allow fungal growth. After an appropriate incubation period, sample (10ml) was withdrawn and centrifuged at 9500 rpm for 20mins in a centrifuge. The cell-free supernatant was used as crude enzyme source. Endoglucanase activity was then revealed by pouring Mandel and Reese agar plates medium supplemented with CMC (1%w/v). After solidification of the medium, 6mm diameter cylindrical probes (wells) were made in the centre of Petri plates with help of cork borer. These wells were filled with crude enzyme of the test organisms. Petri plates were then incubated at 28^{0}C+1 for 2 to 5 days. After incubation, 10 ml of 1% Congo - Red staining solution was added to the plates that were shaken at 50 rev/min for 15 min. The Congo - Red staining solution was then discarded and a 10 ml of 1N NaOH added to the plates and shaken again at 50 rev/min for 15 minutes. Finally, 1N NaOH was also discarded and the staining of the plates was analyzed by noticing the formation of clear or yellowish zones around inoculated well (Teather and Wood 1982). Controls without fungal cultures were conducted simultaneously with test experiments.

Following Congo red screening, fungal isolates with a clear zone of inhibition were subjected to submerge fermentation for their potential ability to produce alkaline cellulase enzyme (endoglucanase and exoglucanase activity). A volume of 100ml Mendel and Reese medium each containing 1% CMC and 1% cellulose powder were distributed into separate 250ml Erlenmeyer flasks. The pH of the medium was adjusted to different alkaline pH (7-11). After autoclaving at 121°C and 15 lb. pressure, the flasks were then

inoculated with spore/mycelia suspension by adding 2ml of the sterile distilled water to freshly grown fungal agar slants (Ramanjaneyulu, *et al.,* 2015). These flasks were incubated at 30°C on a rotary shaker at 120 rpm for 12 days. After every 2 days, culture filtrates were collected and centrifuged at 10,000 rpm for 20mins and the supernatants were used as crude enzyme sources.

5.4 Production of Alkaline Cellulase from Fungal Culture (ENZYME ASSAY)

5.4.1 Assay of Endoglucanase Activity (CMCase)

Endoglucanase activity was measured as described by Mandel (1974). A 0.5 ml. of 1% Carboxymethyl Cellulose (CMC) was mixed 0.5 ml of enzyme sample diluted with citrate buffer (pH 8.0) and incubated for 30 minutes at 50^{O}C. This was followed by the addition of 3ml DNS reagent and boiled for 5 minutes in a water bath. The reaction was stopped immediately and the tubes were cooled in a refrigerator to stop the further reaction and then the results were measured using a spectrophotometer at 540nm. A control tube containing a substrate blank was run concurrently with test samples to the amount of sugar present in the crude enzyme. A glucose standard curve was plotted where the amount of reducing sugar prepared by plotting glucose (mg/ml) against absorbance at 540nm into microgram (µg) of glucose produced during enzymatic reaction. Several dilutions were made of an enzyme in which one dilution released from CMC substrate, slightly more than 0.5mg of the glucose in the reducing sugar assay and other slightly less. A control consisting of heat-denatured enzyme was also run simultaneously with the test to correct any amount of sugar present in the crude enzyme. Enzyme activity was presented in term of International Units (IU), which is micromole (µm) of glucose release /min./ ml.

IU/ml. = mg. glucose X 0.37 X dilution factor

or

$$IU/ml. = \frac{0.185}{\text{Enzyme dilution to give 0.5 mg. glucose}}$$

5.4.2 Assay of Exoglucanase Activity (FPase)

Exoglucanase activity was measured similar to endoglucanase as described by Mandel (1974) with some changes. Here, 0.5ml of enzyme previously diluted in citrate buffer pH (8.0) was added to a test tube containing 1ml citrate buffer and a 1x6cm strips of Whatman #1 filter paper (50mg) was also added into the test tube with different enzyme dilution factor. Following incubation at $50^{O}C$ for 60 minutes, the reaction was stopped by the addition of 3ml DNS reagent and boiled for 5 minutes in a water bath. The tubes were immediately cooled in a refrigerator to stop the further reaction and the absorbance was measured at 540nm. A control tube containing a substrate blank was run concurrently with test samples to the amount of sugar present in the crude enzyme. A glucose standard curve was plotted where the amount of reducing sugar prepared by plotting glucose (mg/ml) against absorbance at 540nm into microgram (µg) of glucose produced during enzymatic reaction. A control consisting of heat-denatured enzyme was also run simultaneously with the test to correct any amount of sugar present in the crude enzyme. Enzyme activity was presented in term of International Units (IU) as Filter Paper Units (FPU) which is micromole (µm) of glucose release /min./ ml.

IU/ml. = mg. glucose X 0.185 X dilution factor

or

$$\text{FPU/ml.} = \frac{0.37}{\text{Enzyme dilution to give 0.5 mg. glucose}}$$

5.5 Identification of Fungal Cultures

Fungal cultures found to be positive alkaline cellulase activity were further identified with help of available literature (Thom and Raper, 1945; Gilman 2001). These fungal cultures were identified based on morphological characteristics using taxonomic guides and standard procedures with the commonly used lactophenol cotton stain. The fungal cultures were further identified based on 18s rRNA sequencing.

5.5.1 DNA Extraction and PCR Amplification

DNA was extracted from the culture isolates. Its quality was evaluated on 1.0 % agarose gel, a single band of high-molecular-weight DNA has been observed. Fragment of 18S rRNA gene was amplified by NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′) primers. A single discrete PCR amplicon band of 1100 bp was observed when resolved on an agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with NS1 and NS4 primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A consensus sequence of 18S rRNA gene was generated from forward and reverse sequence data using aligner software. The 18S rRNA gene sequence was used to carry out BLAST with the ‘nr’ database of the NCBI GenBank database (Zhuang *et al.,* 2012). Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using the RDP database and the phylogenetic tree was constructed using MEGA X (Tamura, *et al.,* 2007).

5.6 Optimization of Physico-chemical and Nutritional Parameters

5.6.1 One Factor at a Time (OFAT) Approach

Important cellulase enzyme production parameters such as pH, nitrogen source, temperature, pH, and incubation time were optimized. The effects of pH (7 to 11), temperature (30 to 60ºC), incubation time (4^{th} 6^{th} 8^{th} 10^{th} and 12^{th}) and were examined. Effect of Carbon source (alkali pre-treated lignocellulosic wastes viz rice straw, wheat straw and sugarcane bagasse). Effect of nitrogen sources (ammonium carbonate, ammonium chloride, ammonium sulphate and sodium nitrate) followed by the effect of percentage concentration (1 to 5%) among nitrogen source will be subsequently tested. All experiments involving these parameters carried out in triplicate with standard deviation while keeping other parameters constant (Remaz *et al.*, 2018).

5.6.1.1 Effect of Initial pH

The effect of pH was determined as per the method below. The pH activity was determined within the alkaline range of 7 to 11. Substrates CMC (1%) for endoglucanase and Whatmann filter paper (50mg) for exoglucanase activity were determined within the ranges as 7, 8, 9, 10 and 11. NaOH and HCl were used to adjust the desired pH (Vega *et al.*, 2012; Prasad *et al.*, 2013).

5.6.1.2 Effect of Temperature

Incubation temperature ranging from 30 to 60ºC were used to observe its effect on the activity of cellulase enzyme based on the work of Basak and Rangan (2018) with modification. The temperature activity was determined by carrying out enzyme production at different temperatures ranges of 30, 40, 50 and 60^0C.

5.6.1.3 Incubation Time

Submerge fermentation of the fungal cultures were conducted for twelve days (4^{th} 6^{th} 8^{th} 10^{th} and 12^{th}) as per the method of Ramanathan *et al.*, (2010) with modification.

At an interval of two days starting from 4th day, crude cellulase enzymes were measure from the initially incubated fungal cultures for estimating enzyme activity.

5.6.1.4 Effect of Different Carbon Sources

The effect of different carbon sources on cellulase production was examined as per the work conducted by Bhavsar *et al.,* (2015) and Nadagouda *et al.,* (2016) with modification. Different carbon sources with rice straw (RS), wheat straw (WS) and sugarcane bagasse (SB) were used for the production of an enzyme as carbon source. The enzyme production was conducted under submerged fermentation at optimum constant pH, temperature and incubation time. The lignocellulosic substrate was alkali pretreated with sodium hydroxide.

5.6.1.4.1 Substrate Preparation

A 200g of each lignocellulosic waste (rice straw, wheat straw and sugarcane bagasse) were dipped in 1000mL of water separately to remove any amount of soluble sugar present in substrates and then dried at 60^0C for 24hrs in electric oven and then chopped into small pieces. The pieces were then grounded in an electric grinder and sieved through 200 mesh sieve. The physically treated substrates were kept at room temperature until use.

5.6.1.4.2 Alkali Pretreatment

The powdered lignocellulosic wastes were treated with alkali sodium hydroxide (NaOH) as per the method described by El-hadi *et al.,* (2014) with slight modification. Dried and powdered lignocellulosic waste (100gm) was treated for 24 hrs with 500 ml of 0.25N NaOH. After treatment, powdered substrates were repeatedly washed with distilled water until the pH of the wash water comes to neutral pH and then dried it overnight at 60^0C.

5.6.1.5 Effect of Nitrogen Sources

The effect of nitrogen sources on the production of enzyme was studied according to the work of Irfan, Nadeem, and Syed (2014). A 1.4g/L Ammonium sulphate (NH_4SO_4), ammonium carbonate (NH_4HCO_3), ammonium chloride (NH_4Cl) and Sodium nitrate ($NaNO_3$) were used for the production of endoglucanase and exoglucanase activity. Following nitrogen source evaluation, the effect of percentage concentration among the best nitrogen has been subsequently tested. All experiments were conducted in triplicates with a standard deviation

5.6.2 Optimization Using Statistical Approach

Design Expert windows vision 6.0.8 portable was the statistical software package used during tabulation and processing process that allows a quick and simple data appraisal. Combined physical and nutritional factors were optimized by response surface methodology using central composite design (CCD). Three major factors selected were ammonium sulphate concentration (1.0 - 5.0%), temperature (30 - 60ºC) and pH (7 - 11) as indicated in **Table 5.1**. A total of 20 different experiments randomly selected by the software were conducted (**Table 5.2**). All experiments are in triplicate and mean enzyme production was used as the variable responses *Y*. Equation indicates the second-order model used in describing the relationship between the independent variable and the response. Experiments are carried out in triplicate while the mean production was used as response variable *Y*. Final RSM predicted response was further validated experimentally (Aanchal *et al.*, 2016) and (de Lima et al., 2010).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Equation… (1)

Key: *Y* is the predicted response parameter, β_0, β_1, β_3, β_{11}, β_{22}, β_{33}, β_{12}, β_{13} and β_{23} are constant regression coefficients of the model, β_0 means intercept term, β_1, β_2 and β_3 are linear coefficients, β_{11}, β_{22} and β_{33} are squared coefficients, β_{12}, β_{13} and β_{23} are interaction coefficients and A, B, C, A^2, B^2, C^2, AB, AC and BC are independent parameters.

5.7 Evaluation of Endoglucanase Enzymes for Deinking Potentials

5.7.1 Selection and Preparation of Waste Paper

A black and white printed with word 'deinking' in a full A4 paper containing 460 words were printed in a xerox printer and used as waste paper in this study. The waste papers were cut into a piece with scissors and soaked overnight in tap water at room temperature. This soaked waste paper was later washed 2 to 3 times in running tap water using 220-wire mesh and disintegrated using a domestic grinder. The obtained pulp slurry was washed with running tap water with the same 220 wire mesh and open dried at $50^{O}C$ (Singh *et al.*, 2012).

5.7.2 Enzymatic Deinking Process

The resultant oven-dried pulp was suspended in 50mM sodium citrate buffer, and enzymatic treatment was conducted using an enzyme dose of CMCase 50ml/10g of oven-dried pulp at $50^{o}C$. To inactivate the enzyme, the pulp suspension was boiled for 10 min. Control assays without enzyme treatment were carried out under identical conditions. Finally, the pulp was washed with tap water through a 220-mesh wire and subjected to flotation for 20 min in presence of 0.1% tween 80 as surfactants and 0.1% $CaCl_2$ as a flotation aid. The flotation device was constructed in our laboratory as per the method of Pala *et al.,* (2004) with modification. A 4L capacity plastic was perforated as flotation reactor attached two air pumps (aquarium pump) containing air stone supplied the flotation tank with air that was connected with an electric switch. At the top of the reactor, the foam was retained and collected. After flotation, hand sheets were prepared according to TAPPI standard from enzyme-treated, as well as control pulp, using a semi-automatic hand sheet maker. The same procedure was conducted conventional deinking by replacing enzyme with 2% Na_2SiO_3, NaOH and 1% H_2O_2 (Soni *et al.,* 2008). The pulp was prepared according to TAPPI standard and sent to the Central Pulp & Paper Research Institute (CPPRI) Saharanpur, 247001 (U.P.) India for the testing of pulp brightness, tensile strength, bursting strength as well as tearing strength.

Table 5.1: Level of independent variables and experimental range from CCD for optimization

Variable	Factors	Range	Level of experimental variables		
			Low (-1)	Medium (0)	High (+1)
A	pH	6-11	6	8.5	11
B	Temperature (^{O}C)	30-60	30	45	60
C	Ammonium Sulphate (%)	1-5	1	3	5

Table 5.2: Experimental design having coded levels of variables used in Central Composite Design with for CMCase and FPase activity

Standard Order	Run	Factor 1 pH	Factor 2 Temperature (^{O}C)	Factor 3 NH_4SO_4conc. (%)
1	16	0 (8.5)	0 (45.0)	0 (3.0)
2	3	1 (11.0)	0 (45.0)	0 (3.0)
3	11	1 (11.0)	-1 (30.0)	1 (5.0)
4	4	-1 (6.0)	-1 (30.0)	-1 (1.0)
5	7	0 (8.5)	0 (45.0)	0 (3.0)
6	20	-1 (6.0)	1 (60.0)	-1 (1.0)
7	5	0 (8.5)	0 (45.0)	0 (3.0)
8	6	0 (8.5)	0 (45.0)	1 (5.0)
9	15	1 (11.0)	-1 (30.0)	-1 (1.0)
10	12	1 (11.0)	1 (60.0)	-1 (1.0)
11	17	-1 (6.0)	1 (60.0)	1 (5.0)
12	8	0 (8.5)	0 (45.0)	0 (3.0)
13	13	-1 (6.0)	-1 (30.0)	1 (5.0)
14	9	0 (8.5)	1 (60.0)	0 (3.0)
15	10	-1 (6.0)	0 (45.0)	0 (3.0)
16	14	0 (8.5)	0 (45.0)	-1 (1.0)
17	19	0 (8.5)	-1 (30.0)	0 (3.0)
18	1	1 (11.0)	1 (60.0)	1 (5.0)
19	18	0 (8.5)	0 (45.0)	0 (3.0)
20	2	0 (8.5)	0 (45.0)	0 (3.0)

5.7.3 Fourier Transformed Infrared Spectroscopy (FTIR)

Fourier transformed Infrared (FTIR) spectrometry was used to study the surface functional groups of the hand sheet from enzyme, chemical and control pulp samples using Attenuated Total Reflectance (ATR) measuring cell. The FTIR spectra of chemical and enzyme deink pulp along with their control were recorded over a range of 4000-400cm^{-1} using 25 scans per sample at a resolution of 4cm^{-1}. Using FTIR database, the spectra were analyzed at the Central Instrumentation Facility (CIF), Lovely Professional University Phagwara.

5.7.4 Scanning Electron Microscopy (SEM)

Morphological and structural changes of deinked and control pulp samples were observed using a Scanning Electron Microscope (SEM). The dried samples were coated with a thin film of gold and observed under different magnification of scanning electron microscope (Lica stereoscan 440) at central instrumentation facility (CIF), Lovely Professional University Phagwara.

5.7.5 Determination of De-Inked Paper Properties

Following pulping and flotation of the enzymatic, chemical and control pulp, the resultant hand sheet was prepared according to TAPPI standard and sent to the Central Pulp & Paper Research Institute (CPPRI) Saharanpur, 247001 (U.P.) India for the testing of pulp brightness, tensile strength, bursting strength as well as tearing strength.

CHAPTER SIX

RESULT AND DISCUSSION

6.1.0 Introduction

Cellulase enzyme can be produced from different microorganisms like bacteria (*Clostridium* spp, *Cellulomonas* spp) and fungi (*Trichoderma, Fusarium, Mucor, Aspergillus* spp) on cellulosic materials. Many microorganisms such as bacteria and fungi produce these cellulase enzymes when they grow on cellulosic materials (Yakubu *et al.,* 2019)**.** The enzyme is relatively costly which has a great advantage for its commercial use. Alkaline cellulase was isolated in the different alkaline environment ranging from alkaline soda Lake to soil. Low enzyme yield and substrate costs are some of the problems associated with cellulase production which mainly affect its large scale production. However, these limitations can be overcome by the application of optimizing parameters controlling enzyme yield. This can be done by either optimizing the physical factors like pH, incubation time and temperature (Ding *et al.,* 2014) or nutrient composition of the media such as carbon and nitrogen source (Zambare 2011; Aanchal *et al.,* 2016). One factor at a time approach (OFAT) is a conventional method for enzyme production which is time-consuming, laborious and overlooks interactions between different variables, leading to misinterpretation of the data obtained. To examine the effects of various factors that influence responses by changing them simultaneously, a statistical approach called Response Surface Methodology (RSM) through Central Composite Design (CCD) can be used to replace one factor at a time (OFAT) approach (Fakhfakh-zouari *et al.,* 2010). The fast depletion of forest resources and its impact on the ecological balance has forced the paper industry to recycle waste paper (Pathak *et al.,* 2010). Current deinking processes depend upon the use of a large amount of environmentally hazardous chemicals such as NaOH, Na_2SiO_3, Na_2CO_3, H_2O_2, chelating agent and surfactants (Zhang *et al.,* 2008). Alternatively, deinking using enzymes is less polluting, energy-saving and also results in lower disposal problems. The present study was aimed at isolating alkaliphilic fungi producing alkaline cellulase enzyme from the

soil and some industrial effluents of Kapurthala and Amritsar district of Punjab with potential usage for the removal of ink from waste paper.

6.2.0 Sampling Sites and Sample Collection

Soils of different villages and industrial effluents from Wahid Sandhar Sugar Ltd Phagwara were selected from Kapurthala district while an industrial effluent of Khanna paper mills in Amritsar region of Punjab were selected as sample sites. Nine out of ten sample sites were located within Kapurthala district. These include soil samples from eight different villages located within the district and Wahid Sandhar sugar Ltd Phagwara. Kapurthala district is reported to be the smallest districts in Punjab in both area and population **(Fig. 6.1.0)**. It is divided into Kapurthala-Sultanpur Lodhi which lies between north latitude $31^0$22' and east longitude $75^0$36' and also Phagwara Tehsil lies at north latitude $31^0$22' and east longitude $75^0$40 as well as 75^0 55'. Phagwara is situated on National Highway No.1 and is industrially developed compared to other Tehsil and districts in Punjab (kapurthala.gov.in). The district is also subdivided in three Tehsil namely Kapurthala, Phagwara and Sultanpur Lodhi with a total area of 1633 km^2. Kapurthala Tehsil has a total area of 909.09km^2, followed by 304.05 km^2 in Phagwara and 451.00 km^2 in Sultanpur Lodhi Tehsil. The soil of the district is mainly water logging and alkaline in nature with majority of the soil in the district have a pH ranges of 6.79 - 9.89 (Singh, *et al.,* 2016). This makes the soil a potential environment for the isolation of alkaliphilic microorganisms. It hosts major crops such as wheat, rice, sugarcane, potato and maize (kapurthala.gov.in).

A total of 17 samples were aseptically collected from 10 designated sites. Twelve (12) of these samples were collected from Kapurthala district while the remaining five samples were collected from Khanna paper mills Amritsar (**Fig. 6.3**). Among 12 samples, one (1) soil sample each was collected from eight villages of Kapurthala district while the other four samples were collected from Wahid Sandhar Sugars Ltd Phagwara (**Fig. 6.4**). These villages include Domeli, Dhoda, Ucha, Randhipura, Natpur, Fattudhinga, Fatehpur, and Samana (**Fig. 6.2**).

Fig. 6.1.0 Map showing District of Kapurthala, Punjab, India (kapurthala.gov.in)

Fig. 6.2.0: Pictorial view of sampling site for soil sample at (a) Samana village and (b) Dohada village in Kapurthala District, Punjab

Fig. 6.3.0: Pictorial view of sampling site of industrial effluent sample from Khanna paper mills Amritsar

Fig. 6.4.0: Pictorial view of industrial effluent sample from Wahid Sandhar Sugars Phagwara

Table 6.1 List of sampling sites and sample collection

S/No	Sampling site	Sample	Sample pH	Sample Code	Address	GPS Location
1	Domeli	Soil	10.34	VDTPDK1	Village of Domeli Phagwara tehsil (Kapurthala District)	Longitude: 31°20.33.654” Latitude: 075° 47.39.276'
2	Dhoda	Soil	9.19	VDTPDK2	About 600m near north east of Jagjit pur village outside of Phagwara-Hspr road	Longitude: 31°19 19.392” Latitude: 075° 49 8.982'
3	Ucha	Soil	9.53	VUTPDK	Near to Ucha pind, in the village of Ucha	Longitude:31°10.592” Latitude: 75° 42 .132'
4	Randhipura	Soil	9.55	VRTPDK	about 1 km before Khairanwali village lift site of Tran-tran	Longitude:1°23.117’ Latitude:075° 17 .821'
5	Fattu Dhinga	Soil	10.69	VFTPDK1	Bolath village 300 m south of Nadal-Bholath road	Longitude:1°32.804’ Latitude:075° 29 .639'
6	Natpura	Soil	9.91	VNTPDK	Old bed of beas river- Nangal Labana village	Longitude:1°37.119’ : Latitude: 075° 28 .290'
7	Fatehpura	Soil	9.04	VFTPDK2	Muradpur Dona village opposite of Rampur village, Kapurthal district	Longitude: 1°15.196’ Latitude 075° 27 .423'
8	Samana	Soil	9.80	VSTPDK	Dakh Darweshpind on Phagwara- Jadiala road	Longitude: 31°11.683” Latitude: 075° 42 .115'
9	Khanna paper mills	Fresh solid	6.54	KPMA/S1	Amritsar	Cash and Carry, NH- 1, Majitha Rd, Opposite Metro, Kishore Nagar, Amritsar, Punjab 143001

10	Khanna paper mills	1 year old solid	6.35	KPMA/S2	Amritsar	Cash and Carry, NH- 1, Majitha Rd, Opposite Metro, Kishore Nagar, Amritsar, Punjab 143001
11	Khanna paper mills	2 years old solid	6.78	KPMA/S3	Amritsar	Cash and Carry, NH- 1, Majitha Rd, Opposite Metro, Kishore Nagar, Amritsar, Punjab 143001
12	Khanna paper mills	Mixed effluent liquid	6.55	KPMA/S4	Amritsar	Cash and Carry, NH- 1, Majitha Rd, Opposite Metro, Kishore Nagar, Amritsar, Punjab 143001
13	Khanna paper mills	Fresh effluent liquid	6.6	KPMA/S5	Amritsar	Cash and Carry, NH- 1, Majitha Rd, Opposite Metro, Kishore Nagar, Amritsar, Punjab 143001
14	Wahid Sandhar sugars Ltd	Fresh sample	7.00	WSSDBS1	Phagwara	G.T. Road, Phagwara, Punjab (144401)
15	Wahid Sandhar sugars Ltd	45 days old	7.80	WSSDB/S2	Phagwara	G.T. Road, Phagwara, Punjab (144401)
16	Wahid Sandhar sugars Ltd	2months old	7.17	WSSDB/S3	Phagwara	G.T. Road, Phagwara, Punjab (144401)
17	Wahid Sandhar sugars Ltd	One year old	6.33	WSSDB/S4	Phagwara	G.T. Road, Phagwara, Punjab (144401)

The samples were taken from the depth of 1-15 inches from the topsoil and sieve with 2mm sieve (Bairagi, *et al.,* 2016). Before collecting soil samples and industrial effluents, the pH of each was measured at the collection sites and recorded **(Table 6.1).**

6.3.0. Isolation of Fungal Cultures

Nutritional and environmental factors such as pH, temperature, moisture, organic carbon and nitrogen play a vital role in the distribution of mycoflora and some of the main factors influencing fungal population and diversity (Kumar *et al.,* 2015). Fungi are said to play an important role in the degradation of lignocellulosic biomass when enzymes are secreted leading to breaking down of the long polysaccharide chains to monomers. In this present investigation, cellulose-degrading alkaliphilic fungi were isolated from alkaline soil and industrial effluents. A total of twenty-seven (27) fungal cultures were isolated within the alkaline pH range of 7 to 11 using Mandel and Reese media supplemented with carboxymethyl cellulose (CMC) and cellulose powder (CP) as carbon sources **(Table 6.2).** The result depicts that potential fungal cultures were found growing at variable alkaline pH from 7 to 11. Research on isolation of alkaliphilic fungi have been reported by various researchers. Eleven fungal strains were reported to produced cellulases in a liquid medium with a potential activity at alkaline pH indicating their alkaliphilic ability (Vega *et al*., 2012). An abundant alkali tolerant species of *Emericellopsis-clade* within the *Acremonium* fungal cluster was reported from alkaline soil of Serbia, Trans-Baikal region (Russia), Aral lake (Kazakhstan) and Eastern Mongolia (Grum-grzhimaylo *et al*., 2013). Reports of 490 alkali tolorant fungal cultures were isolated from natural habitats at pH 11 in Thailand. A total of 324 strains (66%) of these alkaline fungi were screened and found to have good activity when assayed for arabinanase, amylase, potato-galactanase and protease alkaline enzymes (Kladwang *et al*., 2003).

6.4.0 Screening of Alkaline Cellulolytic Fungi

All twenty-seven (27) fungal cultures were subjected to screening using Congo red method. Seven fungal cultures viz (VSTPDKF2, VSTPDKF4, VRTPDKF1, VNTPDKF3, WSSDBS2F1, KPMAS2E1 and KPMAS4F1) fungi have shown a maximum clear hollow zone of hydrolysis (≥10mm) in Mandel and Reese media containing CMC as carbon source **Table 6.3.0.** The fungal culture with code VSTPDKF2 showed the maximum zone of hydrolysis (30mm) followed by WSSDBS2F1 and KPMAS4F1 (21mm each), VSTPDKF4 (15mm), VRTPDKF1 and VNTPDKF3 (14 each) while KPMAS2F1 has the lowest zone of hydrolysis (10mm). The

observation of a clear zone of hydrolysis on the Petri plates is an indication of the presence of potential alkaline cellulase enzyme. A very few reports of fungi that can degrade cellulose to produce cellulase enzymes under alkaline fungi were reported previously (Kumar *et al.,* 2015; Gaur and Tiwari 2015; Ram *et al.*, 2014). The diameter of clear zones within the fungal colonies and control Petri plates with no fungal cultures were also carried out concurrently with test samples. Screening of microorganisms is necessary to determine the screening strategy for detail in cellulase enzyme production for a potential application.

6.5.0 Alkaline Cellulase Enzyme Assay from Fungal Cultures

Production of alkaline cellulase enzymes was conducted on seven alkaline fungal isolates that showed potential hydrolysis zone in Congo red screening. Fungi that produced clear zone are considered as potential cellulase enzyme producers. However, hallows around isolates on solid media may sometimes be due to the presence of membrane-bound hydrolysis which causes the formation of clearing zone when the substrates are being hydrolyzed (Rajendran *et al.*, 2015). Based on this reason, alkaline cellulase enzymes were produced using submerged fermentation techniques. **Table 6.4 and 6.5**, as well as **Figure 6.5 and 6.6** shows the endoglucanase and exoglucanase activity at alkaline pH respectively. Fungal culture VSTPDKF2 has shows the highest endoglucanase (CMCase) activity of 3.52 IU/mL followed by WSSDBS2F1 with CMCase (2.78 IU/mL), VSTPDKF1 (1.11 IU/mL), VNTPDKF2 (0.74 IU/mL), VRTPDKF1 and KPMAS4F1 both have (0.56 IU/mL) and the lowest activity was recorded from isolate KPMAS2F2 with (0.37 IU/mL). Fungal culture VSTPDKF2 has recorded the highest exoglucanase activity of 3.88 FPU/mL followed by WSSDBS2F1 (3.15 FPU/Ml), VRTPDKF1 (1.85 FPU/mL), KPMAS2F2 (0.74 FPU/mL) and the lowest activity was reported both from VNTPDKF2 and KPMAS4F1 of 0.56 (FPU/mL). All enzymatic activity was performed at pH 8. The enzyme activity was found to increase with an increase in incubation days with highest from all isolates found in 8^{th} day incubation. However, a decrease in enzyme activity was observed after 8^{th} day incubation. Similarly, Ramanathan *et al.,* (2010) reported enzymatic activity gradually increase up to 8^{th} day incubation but decline immediately after 8^{th} day fermentation while working on production and optimization of *Fusarium oxysporum.*

Table 6.2: Isolation of alkaline fungi using carboxymethyl cellulose (CMC) and cellulose powder as carbon source

S/NO	CODE	Sample pH	Carbon source	pH 7	pH 8	pH 9	pH 10	pH 11	Total Fungal forms	Fungal Code
1	VDTPDK1	10.34	CMC	-	-	-	-	-	0	
			CP	+	+	+	-	-	2	VDTPDK1F1 and F2
2	VDTPDK2	9.19	CMC	-	-	-	-	-	0	-
			CP	+	+	+	+	-	1	VDTPDK2F1
3	VUTPDK	9.53	CMC	+	+	+	-	-	1	VUTPDKF1
			CP	+	+	+	-	-	1	VUTPDKF2
4	VRTPDK	9.55	CMC	+	+	+	-	-	1	VRTPDKF1
			CP	+	+	+	-	-	1	VRTPDKF2
5	VFTPDK1	10.69	CMC	-	-	-	-	-	0	-
			CP	-	-	-	-	-	0	-
6	VNTPDK	9.91	CMC	+	+	-	-	-	1	VNTPDK2F1
			CP	+	+	-	-	-	1	VNTPDK2F2
7	VFTPDK2	9.04	CMC	-	-	-	-	-	0	-
			CP	-	-	-	-	-	0	-
8	VSTPDK	9.80	CMC	+	+	+	-	-	1	VSTPDKF1
			CP	+	+	+	-	-	1	VSTPDKF2
9	WSSDBS1	7.00	CMC	+	+	+	-	-	1	WSSDBS1F1

			CP	+	+	+	+	-	1	WSSDBS1F2
10	WSSDBS2	7.00	CMC	+	+	+	+	+	1	WSSDBS2F1
			CP	1	1	1	1	0	1	WSSDBS2F2
11	WSSDBS3	7.00	CMC	+	+	+	-	-	1	WSSDBS3F1
			CP	+	+	+	-	-	2	WSSDBS3F2 and F3
12	WSSDBS4	7.00	CMC	+	+	-	-		1	WSSDBS4F1
			CP	+	+	-	-		1	WSSDBS4F2
13	KPMAS1	6.54	CMC	-	-	-	-	-	0	-
			CP	+	-	-	-	-	1	KPMAS1F1
14	KPMAS2	6.35	CMC	+	+	-	-	-	1	KPMAS2F1
			CP	+	+	+	+	+	1	KPMAS2F2
15	KPMAS3	6.78	CMC	+	+	-	-	-	1	KPMAS3F1
			CP	+	+	-	-	-	1	KPMAS3F2
16	KPMAS4	6.55	CMC	+	+	+	-	-	1	KPMAS4F1
			CP	-	-	-	-		0	-
17	KPMAS5	6.6	CMC	+	+	+	-	-	1	KPMAS5F1
			CP	-	-	-	-	-	0	-
Total number of fungal cultures										**27**

Table 6.3: Congo Red screening for endoglucanase (zone of hydrolysis)

S/no	Fungal strain (code)	Maximum growth pH	Zone of hydrolysis (mm)	Control (mm)
1	VDTPDK1F1	7	00	00
2	VDTPDK1F2	8	02	00
3	VDTPDK2F1	8	03	00
4	VSTPDKF1	9	15	00
5	VSTPDKF2	8	30	00
6	VUTPDKF1	8	04	00
7	VUTPDKF2	8	01	00
8	VRTPDKF1	8	14	00
9	VRTPDKF2	7	00	00
10	VNTPDKF1	7	00	00
11	VNTPDKF2	8	14	00
12	WSSDBSIF1	9	05	00
13	WSSDBS1F2	8	04	00
14	WSSDBS2F1	8	21	00
15	WSSDBS2F2	8	06	00
16	WSSDBS3F1	7	00	00
17	WSSDBS3F2	8	03	00
18	WSSDBS3F3	8	02	00
19	WSSDBS4F1	8	01	00
20	WSSDBS4F2	8	05	00
21	KPMAS1F1	7	00	00
22	KPMAS2F1	8	10	00
23	KPMAS2F2	7	00	00
24	KPMAS3F1	8	05	00
25	KPMAS3F2	7	00	00
26	KPMAS4F1	8	21	00
27	KPMAS5F1	7	00	00

Table 6.4.0: Endoglucanase enzyme assay (IU/mL) for the seven fungal cultures

Isolates	4th	6th	8th	10th	12th	Control
VSTPDKF2	1.11±0.18	1.67±0.18	3.52±0.18	1.48±0.00	0.37±0.10	0.00±0.00
VSTPDKF1	0.37±0.00	0.56±0.00	1.11±0.18	0.56±0.00	0.19±0.10	0.00±0.00
VRTPDKF1	0.19±0.18	0.37±0.18	0.56±0.00	0.37±0.10	0.19±0.00	0.00±0.00
VNTPDKF2	0.37±0.00	0.37±0.18	0.74±0.18	0.37±0.00	0.19±0.00	0.00±0.00
KPMAS2F1	0.00±0.00	0.19±0.00	0.37±0.18	0.19±0.00	0.19±0.00	0.00±0.00
KPMAS4F1	0.19±0.18	0.37±0.18	0.56±0.00	0.56±0.00	0.37±0.18	0.00±0.00
WSSDBS2F1	1.48±0.19	1.85±0.18	2.78±0.18	2.22±0.00	0.49±0.10	0.00±0.00

Table 6.5.0: Exoglucanase enzyme assay (FPU/mL) for the seven fungal cultures

Isolates	4th	6th	8th	10th	12th	Control
VSTPDKF2	2.04±0.18	2.41±0.18	3.88±0.18	2.78±0.18	0.37±0.18	0.00±0.00
VSTPDKF1	0.37±0.18	0.56±0.00	0.74±0.18	0.56±0.18	0.37±0.18	0.00±0.00
VRTPDKF1	0.56±0.00	0.74±0.18	1.85±0.18	1.11±0.00	0.37±0.18	0.00±0.00
VNTPDKF2	0.00±0.00	0.19±0.18	0.56±0.18	0.37±0.18	0.19±0.19	0.00±0.00
KPMAS2F1	0.37±0.18	0.56±0.00	0.74±0.00	0.56±0.18	0.19±0.00	0.00±0.00
KPMAS4F1	0.19±0.10	0.56±0.18	0.56±0.18	0.56±0.00	0.00±0.00	0.00±0.00
WSSDBS2F1	1.48±0.19	2.22±0.18	3.15±0.18	2.78±0.18	1.85±0.18	0.00±0.00

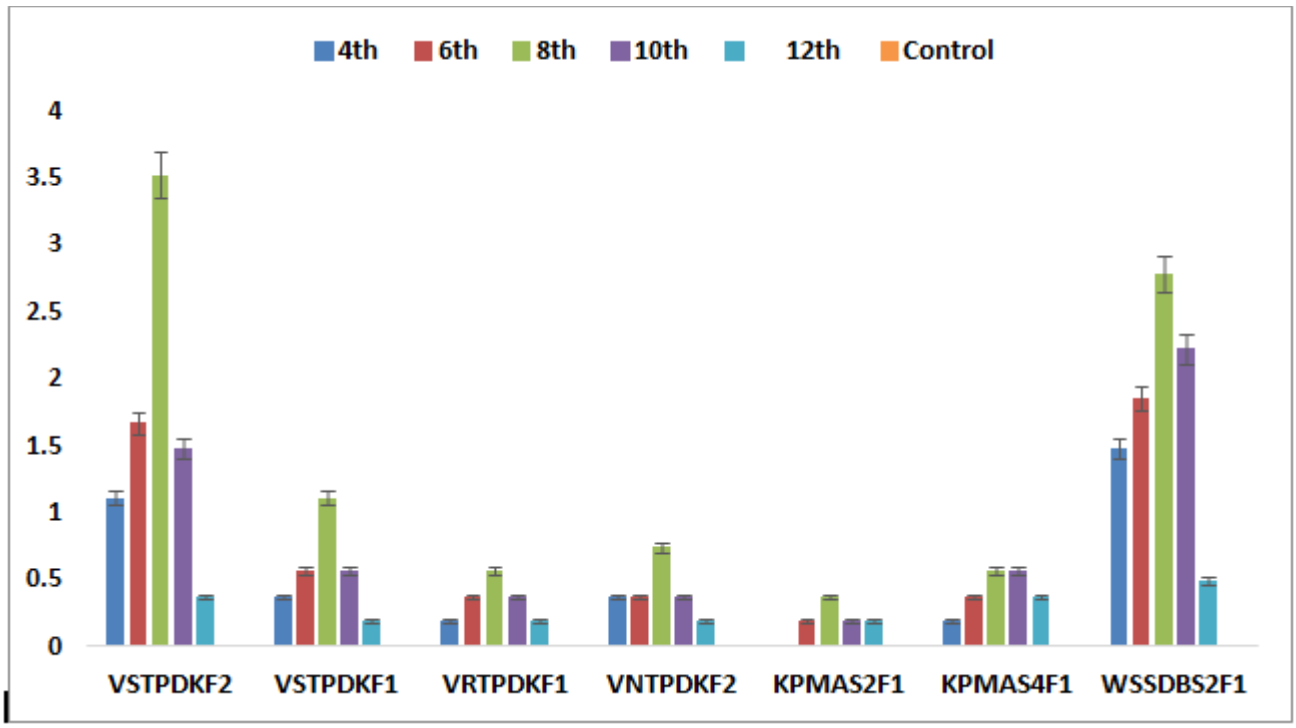


Fig 6.5.0: Endoglucanase enzyme activity (IU/mL) from 7 alkaline fungal cultures at pH 8

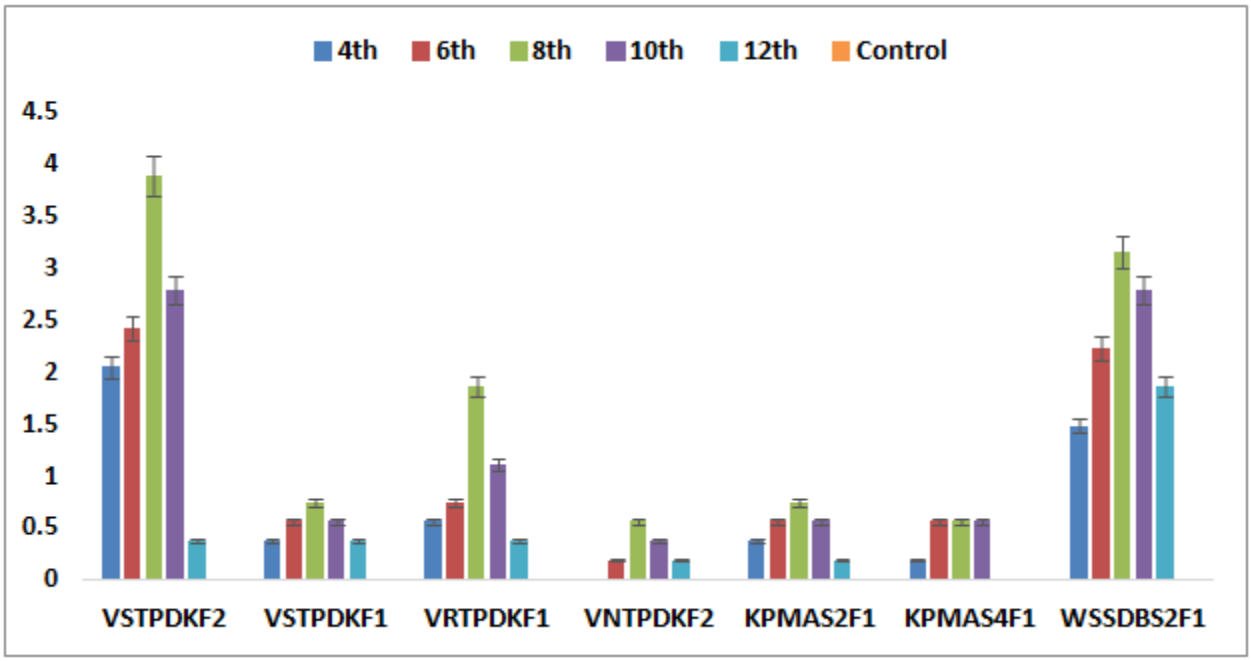


Fig 6.6.0: Exoglucanase enzyme activity (FPU/mL) from 7 alkaline fungal cultures at pH 8

6.6.0 Morphological and Microscopic Identification

Based on colony appearance on Mandel and Reese medium as well as Potato Dextrose Agar medium (PDA), four fungal isolates with high alkaline cellulase activity were identified. Fungal culture of VSTPDKF2 isolated from soil of Samana village appeared as white cottony with dark reverse pigmentation morphologically **(Fig 6.7a)**. Microscopically, it appeared with non-septate conidia, singly, slightly curved macroconidia with a pointed tip **(Fig 6.9a)**. VSTPDKF1 on the other hand, are green to yellowish colony with slight creamy reverse and rapid growth on PDA Petri plate (2-3 days) and moderate growth (4-5 days) on Mandel and Reese media **(Fig. 6.7b)**. Under microscope, VSTDPKF1 isolate appeared septate hyphae, loosely radiate with uniseriate conidial head. Conidiospores are rough and uncoloured as well as smooth conidia **(Fig. 6.9b)**. VRTPDKF1 is green and fast growing fungi morphologically in a Petri plate **(Fig. 6.8a)**. It contains single celled conidia from the brush like conidiaspores when viewed under a compound microscope **(Fig. 6.10a)**. The fungal isolates WSSDBS2F1 found in the decomposing bagasse of sugarcane from Wahid Sandhar Sugar Phagwara is a fast-growing fungus with white coloured colony covering the whole plate and same reverse pigmentation **(Fig. 6.8b)**. Based on microscopic examination, the fungus has broad hyphae which are non-septate with long sporangiospores that terminate in a round spore filled sporangia **(Fig 6.10b)**. Based on colony appearance on Mandel and Rees media as well as Potato Dextrose Agar (PDA) and microscopic examination, the fungal cultures were identified as genera of *Fusarium* (VSTPDKF2), *Aspergillus* (VSTPDKF1), *Penicillium* (VRTPDKF1) and *Mucor* (WSSDBS2F1).

6.6.1 Molecular Identification of Fungal Cultures using 18s rRNA Sequencing

The alkaline fungal culture VSTPDKF2 was isolated from alkaline soil of Samana village in Kapurthala district as explained previously. The fungus was identified as a member of *Fusarium* genera after a microscopic and morphological examination. However, the morphological and microscopic examination may not give actual species for a particular organism. Based on this, molecular 18S rRNA gene identification by Polymerase Chain Reaction (PCR) was conducted at Barcode Bioscience Bangalore India by DNA extraction method **(Fig. 6.11)**. Integrated Transcribed Spacer (ITS1 5′-GTAGTCATATGCTTGTCTC-3′) and (ITS4 5′-CTTCCGTCAATTCCTTTAAG-3′) primers were amplified by 18S rRNA gene region, sequenced and analyzed for similarity among species in NCBI database. A BLAST search

revealed the culture has 97.77% similarity with *F. oxysporum* (Genbank accession number MT102254.1) and a phylogenetic tree was constructed using MEGA X software which the isolate was later assigned as *Fusarium oxysporum* VSTPDKF1 **(Fig. 6.12)**. *Fusarium* species are reported as one of the most important fungal genera with various industrial applications (Hussain, *et al.*, 2018).

Different *Fusarium* species were identified by molecular characterization as reported in many researches. A newly phenol degrading marine strain was identified morphologically, biochemically and using 18S rRNA sequencing analysis. The molecular relationship was compared with other *F. oxysporum* strains between the genes and named as *Fusarium oxysporum* RA (Abedin and Barakat 2013). Kuppusamy *et al.,* (2018) isolated and identified a fungal culture as *Fusarium oxysporum* from a diseased sample of *Allium cepa.* PCR amplification of the 18S ITS regions was conducted using universal ITS primers and the amplified 18S rRNA gene was sequenced and submitted to NCBI database. This was carried out following morphological and microscopic identification. Molecular identification of *Fusarium* cultures was also conducted by amplifying ITS region of the conserved rDNA using ITS1 and ITS4 primers. The eight *Fusarium* isolates F1, F2 and F3 were identified and deposited in NCBI GenBank database as *F. oxysporum* (HM802271, HM802272 and HM802272 respectively), F4 as *F. equiseti* (HQ332532), F6 as *F. proliferatum* (HQ332533) while F5, F7 and F8 are identified as *F. sp* (HQ332534, HQ332535 and HQ332536) respectively (Singh *et al.,* 2016). To isolate and identify *Fusarium* sp in different grains of Egypt, Hussain *et al.,* (2018) used morphological and molecular examination using PCR. The 2 identified species shows a 99% similarity with *F. verticilloides* (Genbank accession number KJ207389.1) and 99% similarity with *Fusarium sp* (Genbank accession number KJ190248.1). In related work, Lin *et al.,* (2012) isolated and identified five fungal strains as *Fusarium* sp based on microscopic examination. These were further analyzed at the molecular stage by PCR amplification using ITS1/ITS4 primers as *Fusarium oxysporum.* The five strains of *Fusarium oxysporum* are H57-1, H38-1, X39-2, H16-1 and X14-6

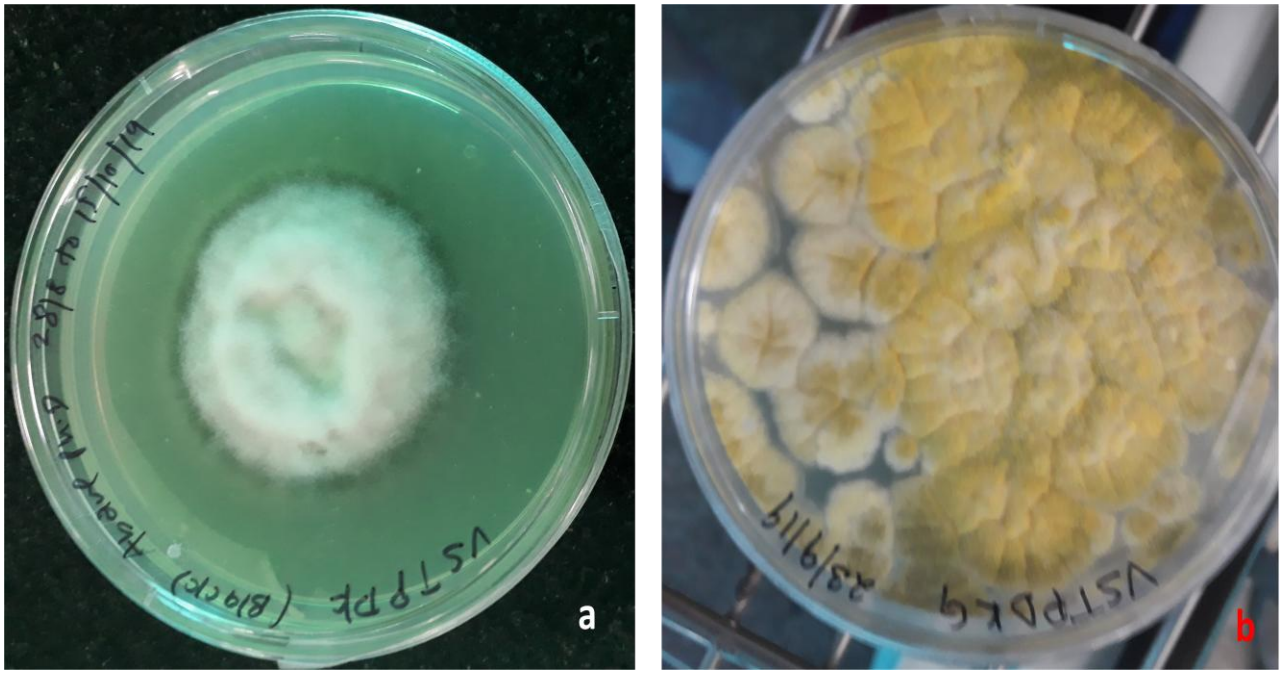


Fig. 6.7.0 Alkaline cellulase producing fungi (a) *Fusarium oxysporum* VSTPDKF2 and (b) *Aspergillus oryzae* VSTPDKF1 when grown on Potato dextrose agar

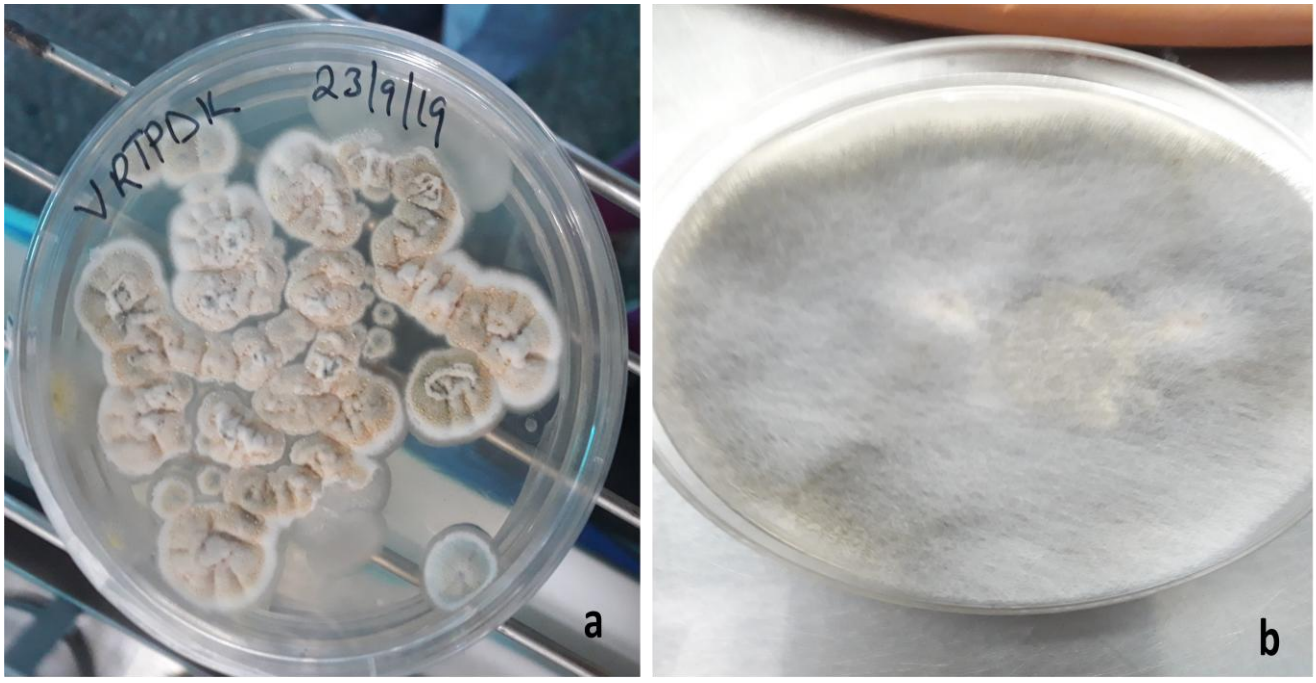


Fig. 6.8.0: **Alkaline cellulase produce fungi (a) *Pencillinium chrysogenum* VRTPDKF1 and (b) *Mucor circinelloides* WSSDBS2F1 when grown on Potato dextrose agar**

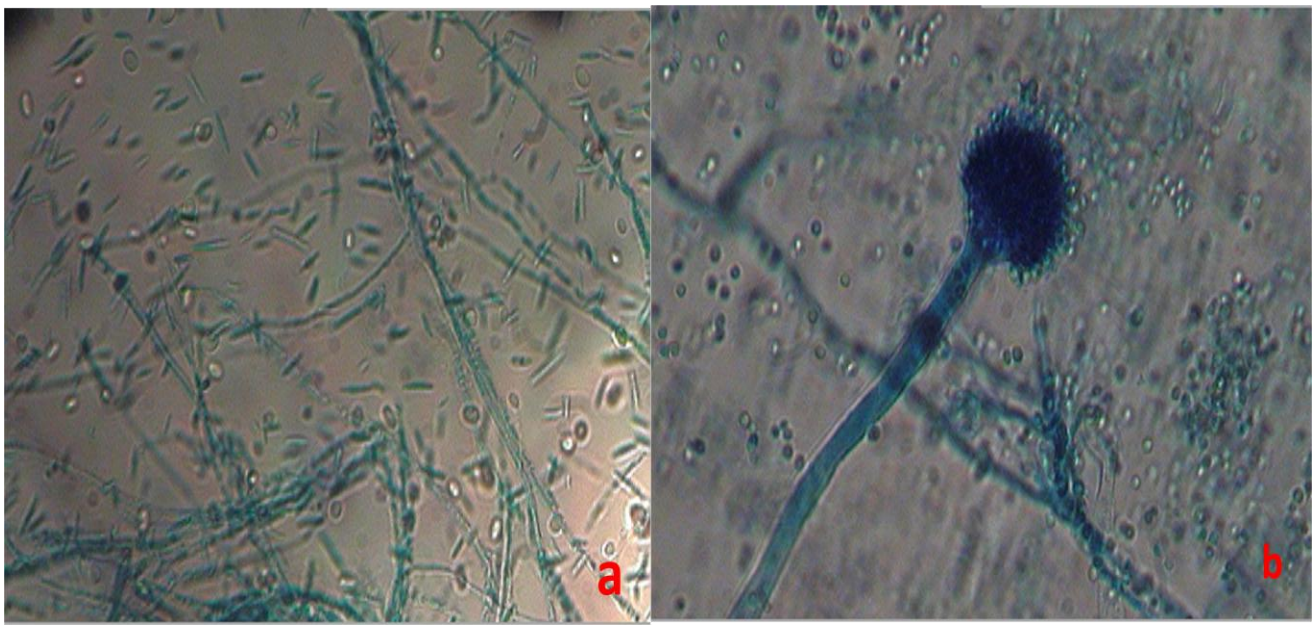


Fig. 6.9.0: Microscopic examination of alkaline fungal isolate (a) *Fusarium oxysporum* VSTPDKF2 and (b) *Aspergillus oryzae* VSTPDKF1 viewed under 40x magnification

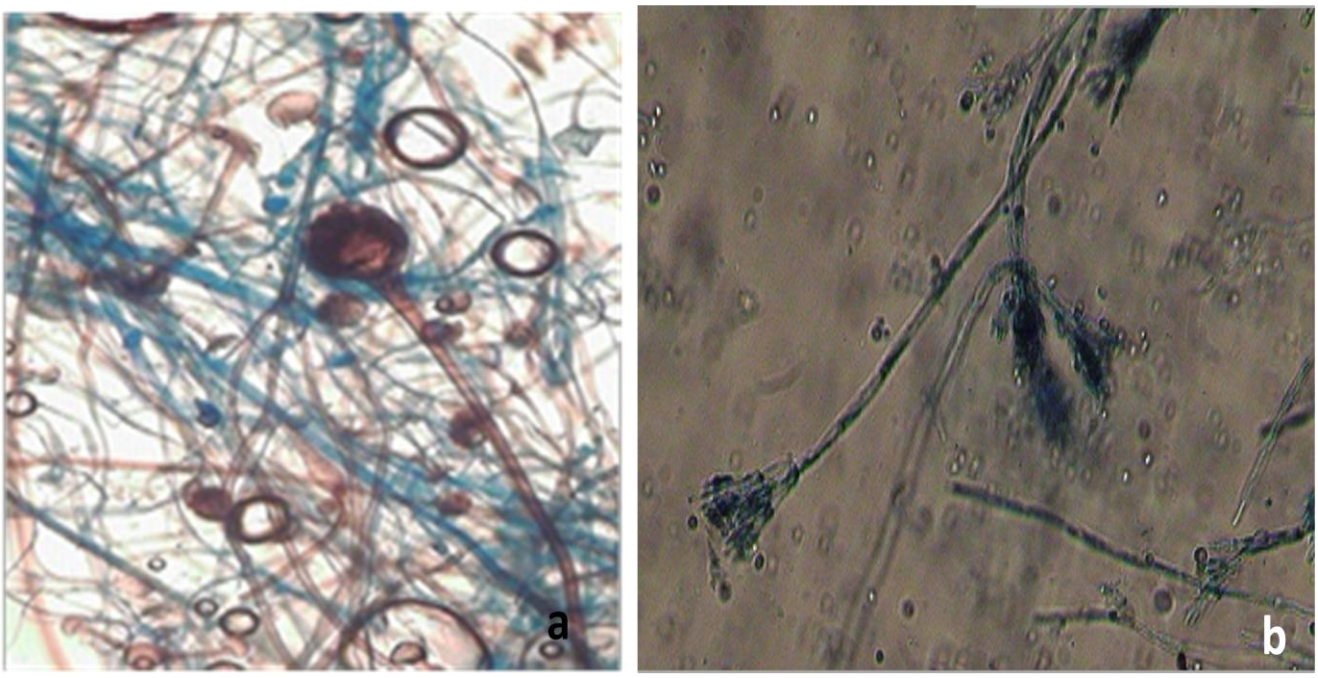


Fig. 6.10.0: Microscopic examination of alkaline fungal isolate (a) *Mucor circinelloides* WSSDBS2F1 and (b) *Penicillinium chrysogenum* VRTPDKF1 viewed under 40x magnification.

gDNA and Amplicon QC data:

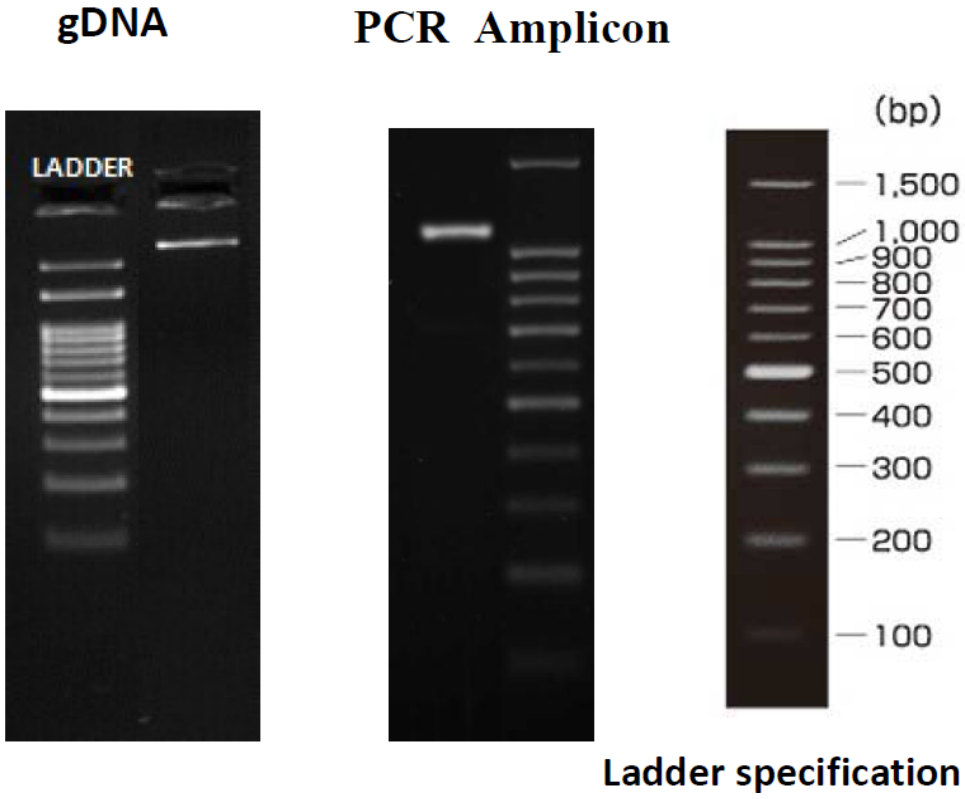


Fig. 6.11.0: PCR of DNA extracted from selected strain. M maker and S sample selected strain

The second alkaline fungus identified was isolated from the same soil of Samana village and designated as VSTPDKF1. The morphological and microscopic examination showed that it belongs to the genera *Aspergillus.* Further identification by 18S rRNA, ITS1, ITS4 and partial rRNA gene sequencing revealed 100% similarity of the isolate *Aspergillus oryzae* (Genbank accession number MH782055.1) based on phylogenetic muscle alignment using MEGA X software as seen in **Figure 6.13**. *Aspergillus niger* sub sp awamori was identified using 18s rRNA sequencing with Genbank accession number KP164968 by Pachauri *et al.,* (2018) when a cellulase producing *Aspergillus* species were isolated from wood chips. Kebeish and El-sayed (2014) identified *A. flavipes, A. carneus, A. flavus, A. tamari, A. oryzae* and *A. parasiticus* based on morphological and physiological features from Egyptian soil. Their relative molecular identity was revealed when primers of 18s rRNA and internal transcribed spacers (ITS1) amplification were combined to give the same polymerase chain reaction (PCR) amplicon. Among the six isolates, *Aspergillus flavipes* and *A. carneus* showed PCR products on agarose gel when ITS2 was used indicating their actual morphological and biochemical similarities. *A. flapices* (Genbank accession number JF831014) indicated a typical identity and deposited in Genbank as *A. flapices* when ITS1-5.8S-ITS2 region was sequenced, blast and aligned with the database. Similar method was used to identify a fungal isolate F3 as *Aspergillus fumigatus* by M/S Bangalore India including ITS1, 5.8S rRNA, ITS2, partial 28S rRNA gene sequence. The identified isolate was tentatively assigned as *Aspergillus fumigatus* NITDGPKA3 with NCBI accession number as JQ046374 (Sarkar and Aikat 2014). Different fungal isolates from the genus *Aspergillus* were isolated, identified morphologically and using 18S rRNA in many papers. Sridevi *et al.,* (2016) isolated and identified a fungal isolate as *A. niger* with accession number KT727925. This potential xylanase enzyme producer was isolated from forest soil of Tirumala, AP India and its crude enzymes were observed for biobleaching of waste paper. In a related development, fungal isolate F-3 was identified and named *Aspergillus sp.* strain F-3 with Genbank accession number GQ149340 (Yang, *et al.,* 2011). A high cellulase producing fungal isolate was also identified as *A. niger* with a BANKIT ID as 2002596 (Harinathan, *et al.,* 2017).

The identification process of the third fungal culture VRTPDKF1 isolated from the soil of Randhipura village in Kapurhtala district was also conducted. Morphological and microscopic examination showed that it belongs to the genera *Penicillium.* However, 18S rRNA, ITS1, ITS4 and partial rRNA gene sequencing revealed the isolate has a 99.71% similarity with *Penicillium*

chrysegenium (GenBank accession number KX580630.1) based on phylogenetic muscle alignment using MEGA X software as seen in **Figure 6.14**. This was similarly reported by different researchers. Ibrahim, *et al.,* (2016) reported a fungal strain SAF6 with high cellulase activity. Molecular identification using 18S rRNA gene sequencing technique was used and the BLAST search revealed it as *Penicillium sp* with 99% similarity. The isolate was named as *Penicillium sp* SAF6-EGY with GenBank accession number KM222497. Jung et al 2015 isolated a new strain *Penicillium* sp GT2 from the soil while comparing its cellulase activity with a commercial strain of *Trichoderma reesei* RUT-C30. 18S rRNA sequences of this *Penicillium* sp GT2 from the complete sequencing by a BLAST search showed it 99% similarity with *Penicillium decumbens* (Genbank accession number KC960012). Both morphological and molecular analysis of fungal isolate L-06 was conducted for identification. 18S rDNA sequence of the isolate showed 99% similarity with *Penicillium decumbens* and assigned EU273880 as Genbank accession number (Liu, *et al.,* 2008).

The last alkaline cellulase producing fungal culture WSSDBS2F1 was isolated from decomposing sugarcane bagasse of Wahid Sandhar Sugar Ltd Phagwara. Microscopic, morphological and molecular identification revealed the isolate to have 96.84% similarity with *Mucor circinilloides* (GenBank accession number HQ845293.1) and then assigned to be *Mucor circinilloides* WSSDBS2F1. The neighbor-joining tree depicting the phylogenetic relationship of the isolate with related species were conducted after muscle aligning with MEGA X software **(Fig 6.16)**. A similar fungus marked as S3 was isolated from mining tailing in Anshan for Pb bioremediation. The internal transcribed spacer (ITS) sequencing revealed that the isolate belongs to *Mucor circinelloides* (Sun et al., 2017). Another fungal isolate Q531 was identified when 18S rDNA sequence was conducted and submitted to Genbank with accession number KU523400. BLAST search result revealed the sequence has a 99% similarity with *M. circinelloides* (Qiao *et al.,* 2018). El-hersh *et al.,* (2017) isolated a potential fungal isolate of high protease production. Morphological and microscopic examination as well as 18S rRNA sequence confirmed the isolate as *Mucor mucedo* strain SEE1 and deposited in the Genbank database as KP736529. Similarly, a filamentous fungi were isolated and identified as *Mucor* sp GY-1 when the neighbor-joining tree depicting the phylogenetic relationship of the isolate with *Mucor* sp based on 18S rRNA gene sequencing (Wang *et al.,* 2015).

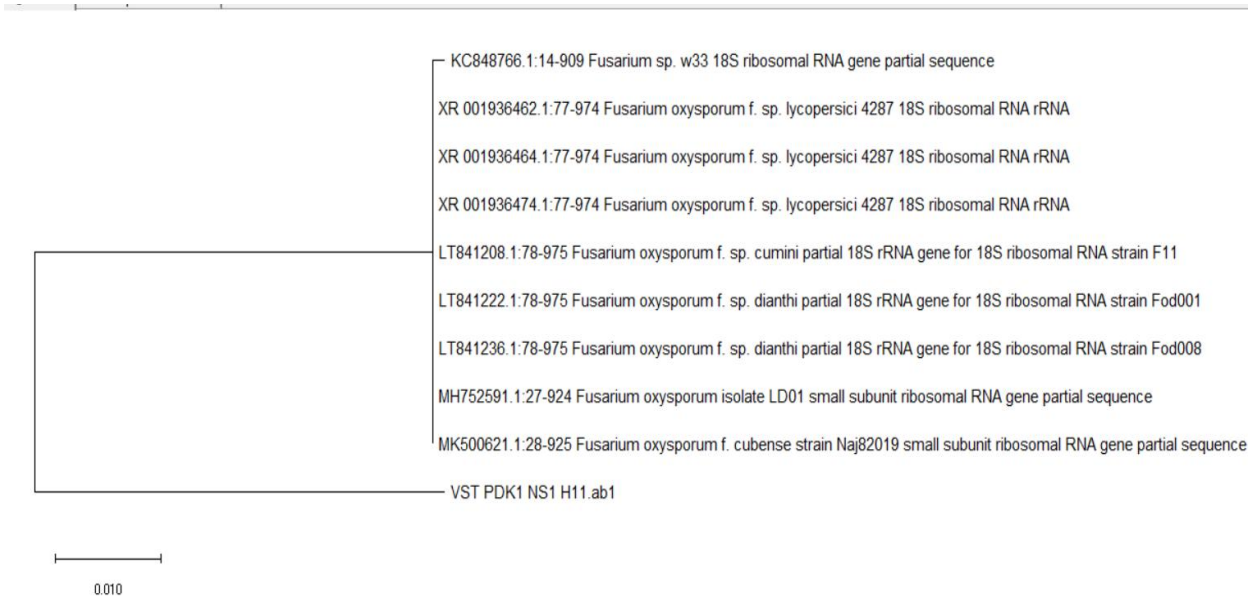


Fig. 6.12.0: Phylogenic tree based on ITS region gene sequences showing relationship between fungal strain VSTPDKF2 and the closest species of *Fusarium oxysporum* based on the neighbor-joining analysis.

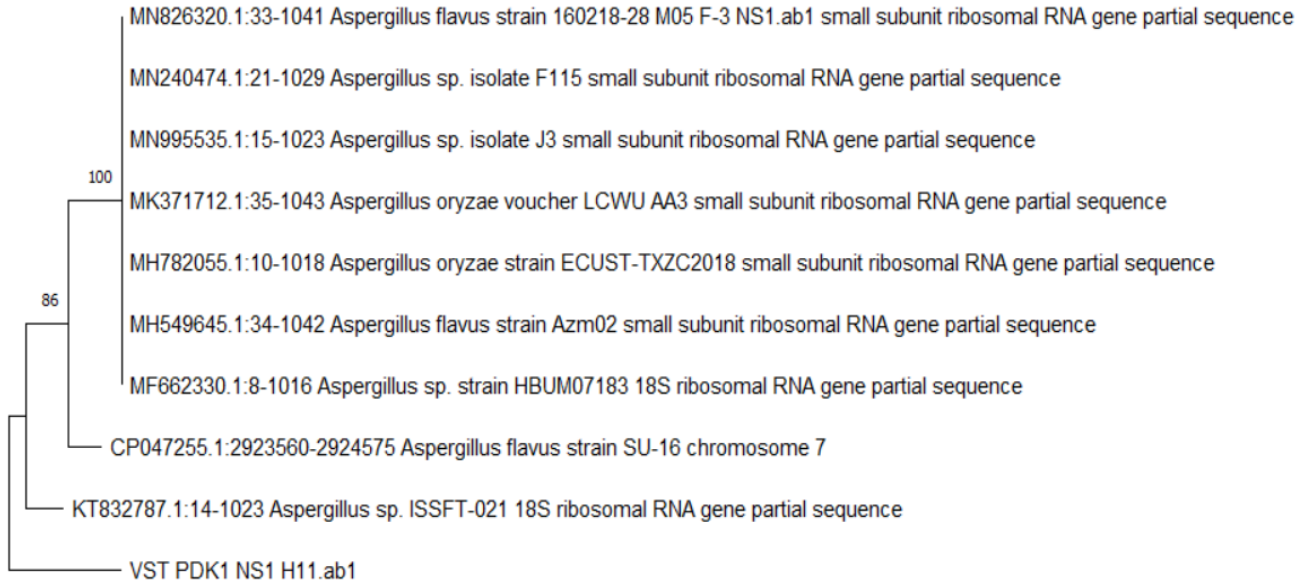


Fig. 6.13.0: Phylogenic tree based on ITS region gene sequences showing relationship between fungal strain VSTPDKF1 and the closest species of *Aspergillus oryzae* based on the neighbor-joining analysis

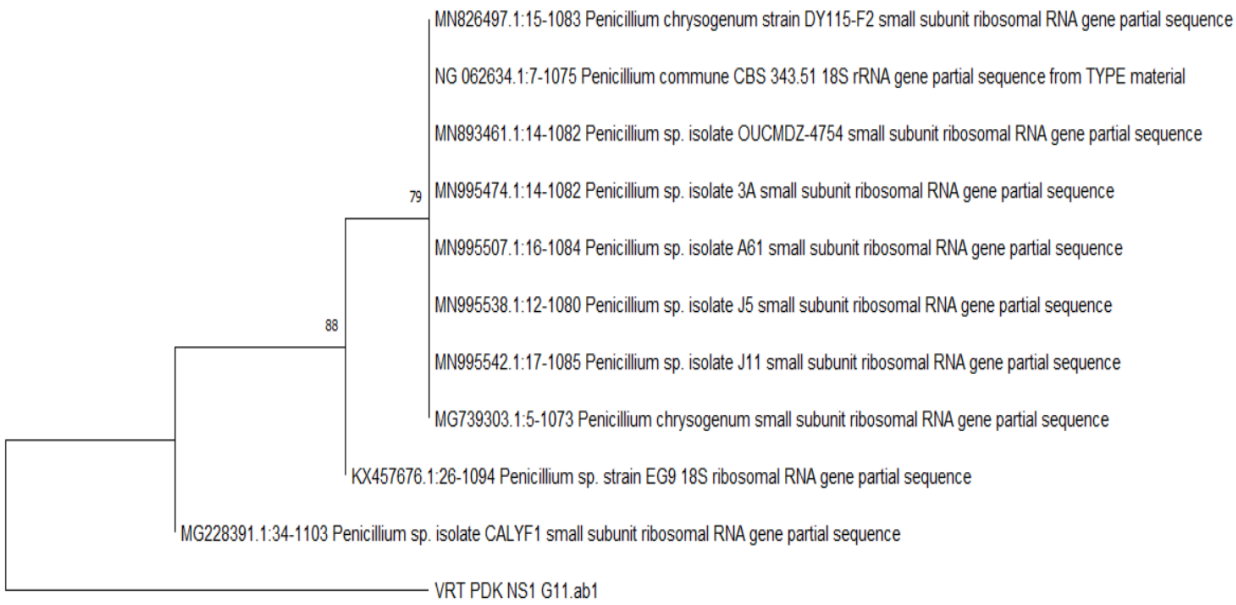


Fig. 6.14.0: Phylogenic tree based on ITS region gene sequences showing relationship between fungal strain VRTPDKF1 and the closest species of *Penicillium chrysogenum* based on the neighbor-joining analysis

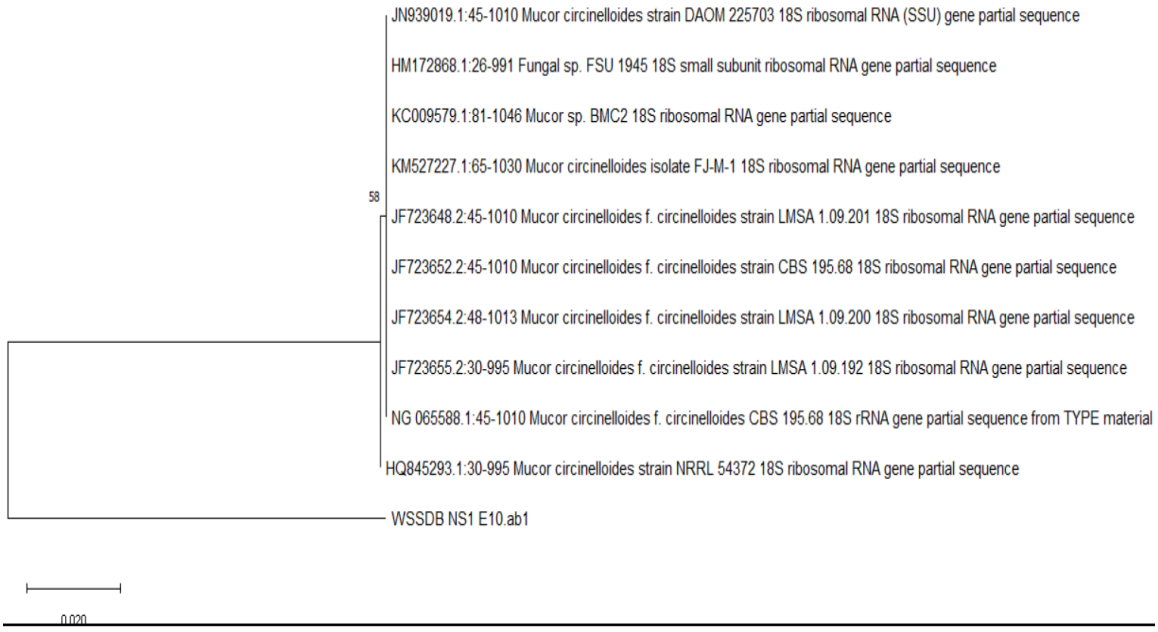


Fig. 6.15.0: Phylogenic tree based on ITS region gene sequences showing relationship between fungal strain WSSDBS2F1 and the closest species of *Mucor circinelloides* based on the neighbor-joining analysis

6.7.0 Optimization of Culture Conditions using One Factor at a Time (OFAT) Approach

Two alkaline fungal cultures with *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 were further selected for evaluation on optimizing the culture conditions using OFAT and RSM. Effect of pH, temperature, incubation time, different carbon sources, nitrogen sources and ammonium sulphite concentration were selected. pH, temperature and ammonium sulphite concentration were further optimized using Central Composite Design in RSM software.

6.7.1 Effect of Initial pH

A good essential physical factor influencing microbial growth and metabolism is the pH (Abatenh, *et al.,* 2017). It is one of the most important physical parameters where an increase or decrease may affect microbial growth and enzyme production (Asira, 2013). Being the cardinal factor of our research, the effects of pH on fungal growth and cellulase enzyme production was studied between the ranges of 7 to 11. It was observed that enzyme activity decreases with an increase in pH value. In this research, optimum alkaline cellulase activity from *F. oxysporum* VSTPDKF2 in both CMCase and FPase (3.33 IU/ml and 3.89 FPU/ml) at pH 7, followed by pH 8, (2.96IU/mL and 2.78FPU/mL), pH 9 (0.56 IU/mL and 1.11 FPU/mL), pH 10 (0.19 IU/mL and 0.37 FPU/mL) and the lowest enzyme activity of 0.19 from both enzymes at pH 11 **(Fig. 6.16)**. In the case of *M. circinelloides* WSSDBS2F1, both CMCase and FPase was found have highest enzyme activity at pH 7 (2.78 IU/ml and 3.33 FPU/ml), followed by pH 8, (2.59 IU/mL and 2.78FPU/mL), pH 9 (1.85 IU/mL and 1.85 FPU/mL), pH 10 (0.19 IU/mL and 0.56 FPU/mL) and the lowest enzyme activity at pH 11 was found only FPase (0.19 FPU/mL) and while no activity was found in CMCase at pH 11 **(Fig. 6.17)**. However, since a significant amount of enzyme activity and stability was recorded across an alkaline environment (8-10). It is believed that this enzyme activity at the alkaline environment is an indication that these isolated *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 can produce alkaline cellulase. *M. circinelloides* NRRL 26519 was reported to be a fairly and highly active in production of endoglucanase over a wider range of pH 3.5-7.5 by Saha (2004). Our result of *M. circinelloides* WSSDBS2F1 is active at alkaline pH. A similar result at the alkaline environment from different fungal strains were reported by (Vega *et al.,* 2012; Prasad *et al.,* 2013; Muhammad *et al.,* 2011; Dutta *et al.,* 2018).

6.7.2 Effect of Temperature

One of the essential physical factor affecting the survival and growth of microorganisms is temperature. Any specific enzyme-mediated degradation process will have an optimum temperature (Abatenh *et al.,* 2017). The temperature for fungal growth and alkaline cellulase production was measured between ranges of 30ºC to 60ºC as shown in **Figure 6.18 and 6.19**. The figure clearly explained that *F. oxysporum* VSTPDKF2 have shown optimum growth temperature for both CMCase and FPase enzyme activity at 30^{O}C (3.50 IU/ml and 3.70 FPU/ml) followed by 40^{O}C (3.15 IU/mL and 3.50 FPU/mL), 50^{O}C (1.85 IU/mL and 0.37 FPU/mL) with least activity at 60^{O}C (0.19 IU/mL and 0.00 FPU/mL) respectively. The optimum CMCase and FPase activity from alkaline *M. circinelloides* WSSDBS2F1 based on increase in temperature was recorded at 30^{O}C (3.32 IU/ml and 3.52 FPU/ml) and then decrease across different temperature ranges 40^{O}C, 50^{O}C and 60^{O}C (2.96 IU/mL and 2.76 FPU/mL), (0.93 IU/mL and 0.19 FPU/mL) and (0.00 IU/mL and 0.19 FPU/mL) respectively. This result is in agreement with that of Jahangeer *et al.,* (2005) and Panagiotou *et al.,* (2003) were they also found an optimum temperature of cellulase enzyme production at 30^{O}C and 33^{O}C from *Aspergillus niger* and *F. oxysporum* respectively. However, the result was in parallel with work of Basak and Rangan (2018) and Remaz, (2018) that produced optimum cellulase from *F. oxysporum* and *Aspergillus niger* at the temperature of 60^{O}C and 50^{O}C respectively. Ravindran *et al.,* (2010) also has reported optimum CMCase from *Chaetomium sp* at 50^{O}C in different alkaline pH using agricultural and industrial waste as substrate. Despite having an optimum production temperature of 30^{O}C, higher temperature values also yield a significant amount of both CMCase and FPase. Thus, cellulase enzyme produced *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 can show enzymatic stability at high temperature.

6.7.3 Effect of Incubation Time

Production of cellulase enzyme was determined based on incubation period by inoculating the flasks in different incubation days (4^{th} 6^{th} 8^{th} 10^{th} and 12^{th}). Following incubation, it was observed that initial increase in fermentation day increase enzyme activity but later declined after reaching peak incubation day at 8^{th} day. As indicated in **(Fig. 6.20)**, optimum CMCase and FPase activity of 3.52 IU/ml and 3.88 FPU/ml were obtained from *F. oxysporum* VSTPDKF2 on 8^{th} day incubation. This was followed by 10^{th} day (1.48 IU/mL and 2.78

FPU/mL), 6th day (1.16 IU/mL and 2.41 FPU/mL), 4th day (1.11 IU/mL and 2.04 FPU/mL) with lowest at 12th day (0.37 IU/mL and 0.37 FPU/mL) for CMCase and FPase activity respectively. Similarly, 2.78 IU/ml and 3.88 FPU/ml CMCase and FPase activity was the maximum activity recorded on 8th day from *M. circinelloides* WSSDBS2F1 **(Fig. 6.21).** The second highest activity was found on 10th day as 2.78 IU/mL and 2.22 FPU/mL, then 6th day with 2.41 IU/mL and 1.85 FPU/mL followed by 4th day 2.04 IU/mL and 1.48 FPU/mL while lowest activity was found on 12th day with 0.37 IU/mL and 0.49 FPU/mL for CMCase and FPase activity respectively. This result comply with the work of Ramanathan *et al.,* (2010) where a maximum CMCase (1.92±0.005) and FPase (1.34±0.003) were produced from *Fusarium oxysporum* after eight days of incubation. However, maximum production at low incubation time was also reported when El-hadi *et al.,* (2014) found a maximum enzyme at 9hrs incubation time for the production of CMCase on *Aspergillus hortai*

6.7.4 Effect of Different Natural Lignocellulosic Carbon Source

Lignocellulose is a major component of plants produced due to photosynthetic activity every year. The chemical nature of lignocelluloses made this complex compound a potential carbon source for the production of this industrial enzyme. Three different types of agricultural wastes were selected for evaluating the production of extracellular alkaline cellulase on alkali pretreated (NaOH) lignocellulolytic substrates. Lignocellulolytic substrates viz Rice straw (RS), wheat straw (WS) and sugarcane bagasse (SB) were selected. These agro lignocellulosic wastes were used to substitute carboxymethyl cellulose (CMC) and cellulose powder as sole sources of carbon in the fermentation media. The optimum condition of the fermentation was maintained at pH 8, temperature 30 and 8th day incubation period for the evaluation of both enzymes. The depicted maximum CMCase (2.78 IU/ml) and FPase (3.70 FPU/ml) activity from *F. oxysporum* VSTPDKF2 was found on rice straw. This was followed by sugarcane bagasse (2.41 IU/mL and 3.33 FPU/mL) and lowest was observed from wheat straw (0.93 IU/mL and 1.85 FPU/mL) **(Fig. 6.22)**. Meanwhile, optimum CMCase (3.70 IU/ml) and FPase (3.89 FPU/ml) activity from *M. circinelloides* WSSDBS2F1 was shown on sugarcane bagasse. This was followed by rice straw (2.41 IU/mL and 3.33 FPU/mL) and lowest on wheat straw (2.04 IU/mL and 2.22 FPU/mL) for CMCase and FPase respectively (**Fig 6.23)**. This activity was in accordance with work of Sasi *et al.,* (2012) were maximum CMCase of 0.128g/ml was found in rice bran followed by wheat brand (0.097g/ml) and sugarcane bagasse (0.019g/ml) respectively.

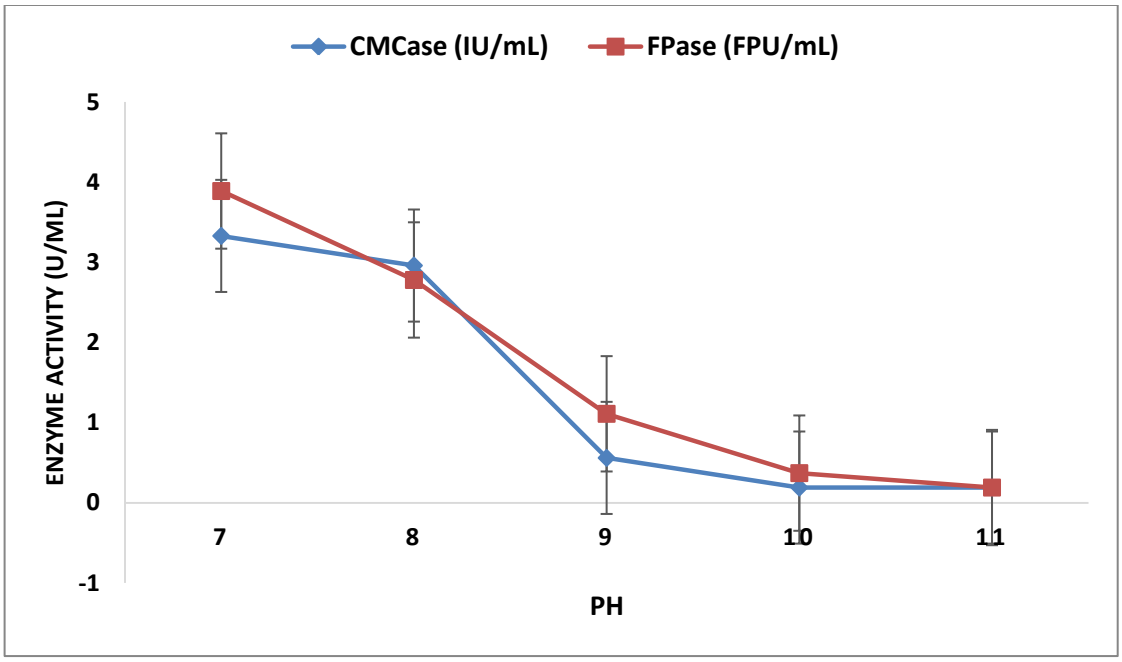


Fig. 6.16.0: Effect of initial pH on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *F. oxysporum* VSTPDKF2

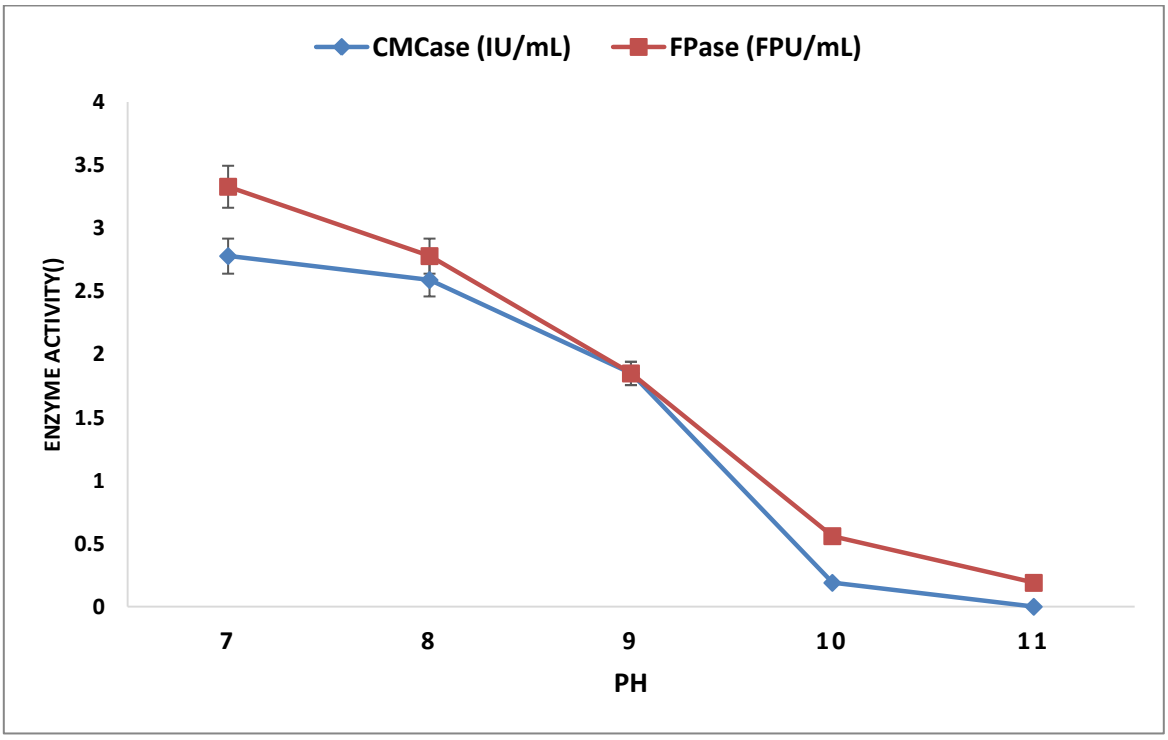


Fig. 6.17.0: Effect of initial pH on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *M. circinelloides* WSSDBS2F1

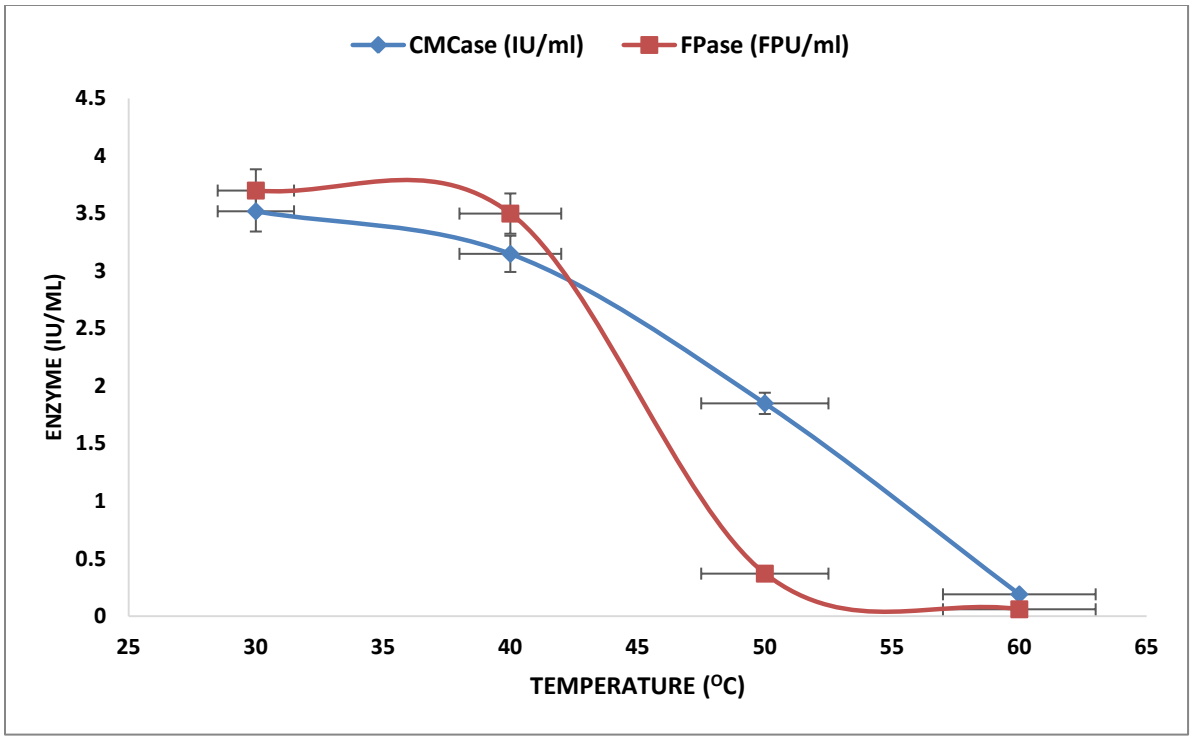


Fig. 6.18.0: Effect of temperature on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *F. oxysporum* VSTPDKF2

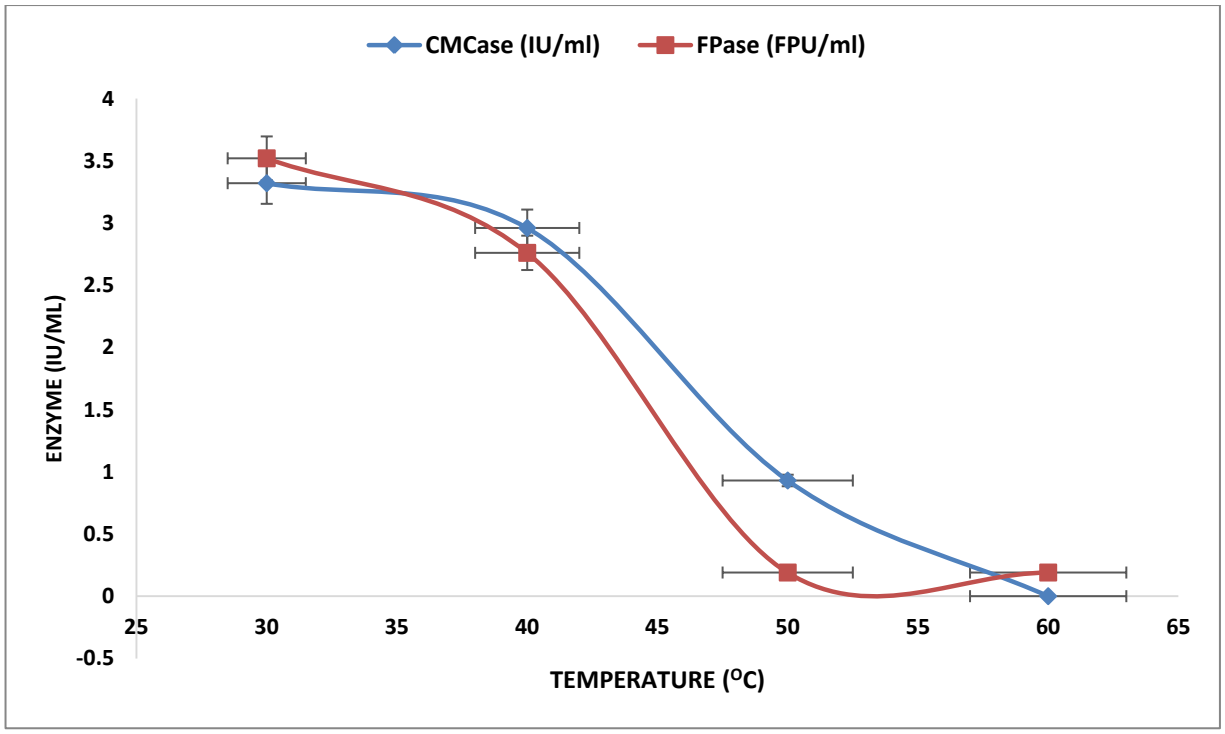


Fig. 6.19.0: Effect of temperature on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *M. circinelloides* WSSDBS2F1.

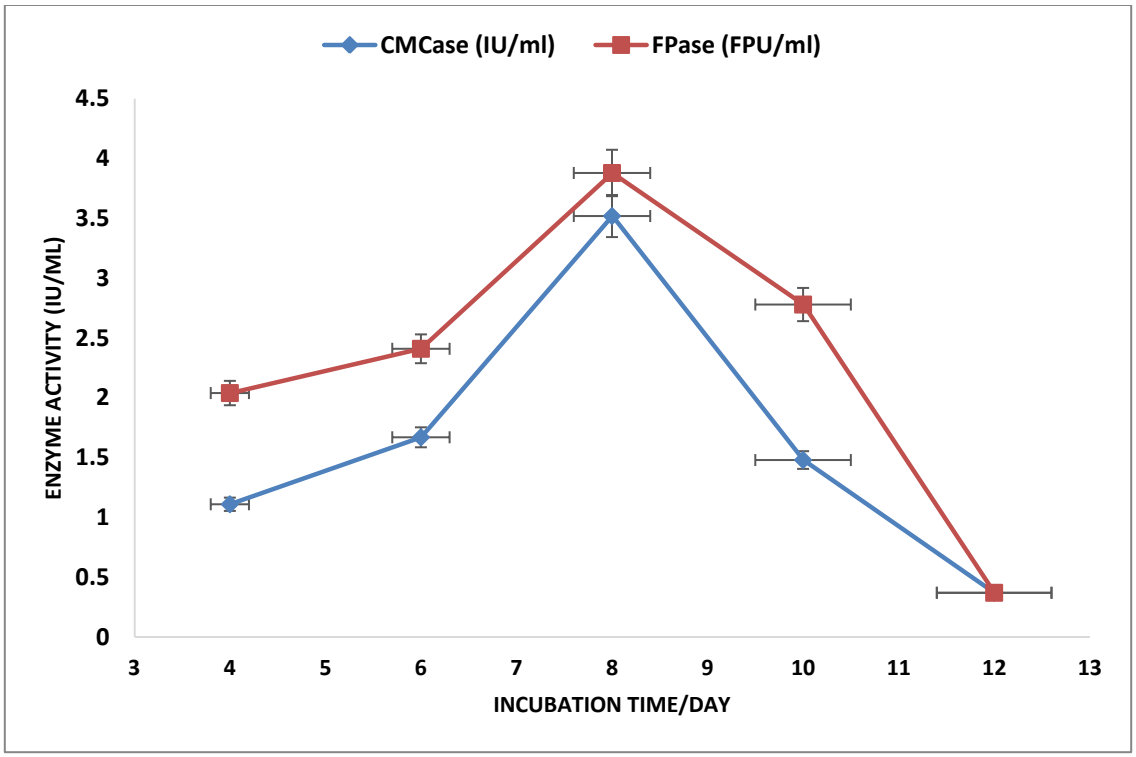


Fig. 6.20.0: Effect of incubation time on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *F. oxysporum* VSTPDKF2

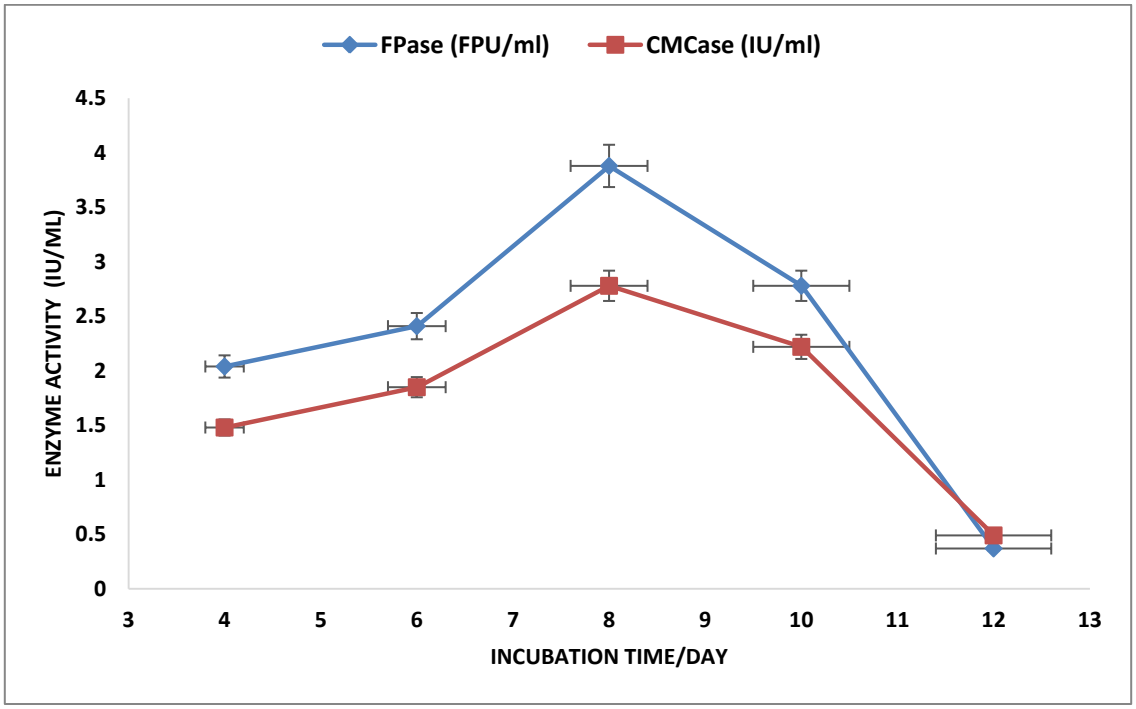


Fig. 6.21.0: Effect of incubation time on the production of endoglucanase (CMCase) from *M. circinelloides* WSSDBS2F1

Bhavsar *et al.,* (2015) also reported a maximum CMCase production of 7.4 IU/ml from banana stem followed by rice straw 5.4U/ml using fungal cellulase. In another research, cellulose and wheat bran were found as the best carbon source in the production of maximum CMCase and FPase from alkaline *Stachybotrys microspora* between the pH 7-8 then glucose, CMC and lactose (Hmad *et al.,* 2014). Based on the amount of enzyme evaluated, it was found that lignocellulolysic wastes that are abundant in Punjab region been an agricultural hub, can be used as a substrate for large scale production of alkaline cellulase enzyme.

6.7.5 Effect of Nitrogen Source

Nitrogen source is another important nutritional factor influencing microbial growth and cellulase production. Different nitrogen sources may have inhibitory or stimulatory effects on fungal growth and cellulase production. Four different inorganic nitrogen sources which include ammonium sulphate (NH_4SO_4), ammonium carbonate (NH_4HCO_3), ammonium chloride (NH_4Cl) and Sodium nitrate ($NaNO_3$) were used as a source of nitrogen **(Fig. 6.24 and 6.25)**. The result clearly shows that *F. oxysporum* VSTPDKF2 had a maximum enzyme activity of 3.50 IU/ml and 3.70 FPU/ml for CMCase and FPase respectively when grown on NH_4SO_4. This was followed by NH_4Cl (2.78 IU/mL and 1.87 FPU/mL), NH_4HCO_3 (1.85 IU/mL and 1.29 FPU/mL) and lowest activity in $NaNO_3$ with 1.11 IU/mL and 1.29 FPU/mL. Result of *M. circinelloide*s WSSDBS2F1 shows optimum CMCase (3.52 IU/mL) and FPase 3.50 (FPU/mL) on NH_4SO_4 and followed by NH_4Cl (2.59 IU/mL and 2.04 FPU/mL), NH_4HCO_3 (1.29 IU/mL and 1.48 FPU/mL) and lowest from $NaNO_3$ (1.11 IU/mL and 1.11 FPU/mL) respectively. The results on the effect of different nitrogen sources obtained in this research were similar with one reported by Sasi *et al.,* (2012) who found that ammonium sulphate increases the amount of cellulase enzymes produced from *Aspergillus flavus* while Vyas *et al.,* (2005) reported an optimum cellulase when ammonium sulphate was used as a nitrogen source. However, it disagrees with the work of El-hadi *et al.,* (2014) and Irfan *et al.,* (2014) were ammonium nitrate was reported to have maximum cellulase activity followed by ammonium sulphate. Urea was also found to be the best nitrogen source on the production and optimization of cellulase enzyme from *F. oxysporum* (Ramanathan *et al.,* 2010).

6.7.6: Effect of Ammonium Sulphate Concentration

Effect of various ammonium sulphate concentrations on alkaline cellulase production was investigated. The result obtained indicated that 3.0% was the optimum concentration supporting cellulase production form both alkaline *F. oxysporum* VSTPDKF2 CMCase and FPase (3.70 IU/ml and 3.89 FPU/ml) **(Fig. 6.26)**. This was followed by 2.0% concentration (3.52 IU/mL and 3.52 FPU/mL), 1% (3.33 IU/mL and 3.52 FPU/mL), 4% (2.22 IU/mL and 2.04 FPU/mL) with lowest activity from 5% concentration (1.29 IU/mL and 1.18 FPU/mL) for both CMCase and FPase respectively. Alkaline *M. circinelloides* WSSDBS2F1 produced optimum CMCase and FPase activity (3.52 IU/ml and 3.33 FPU/ml) by addition of ammonium sulphate concentration to 3% **(Fig. 6.27)**. This was followed by 2% (3.33 IU/mL and 2.96 FPU/mL), 1% (2.78 IU/mL and 2.59 FPU/mL), 4% (2.22 IU/mL and 2.04 FPU/mL) and the least activity when concentration was increased to 5% (2.04 IU/mL and 1.11 FPU/mL) on the production of CMCase and FPase respectively. Increase or decrease in ammonium sulphate from the optimum concentration will lower the rate of enzyme production. This result indicated that ammonium sulphate is one of the best nitrogen sources for alkaline cellulase production. This result is similar to the result obtained by Vyas *et al.,* (2005) and Sasi *et al.,* (2012) where ammonium sulphate was reported to be optimum.

6.8 Central Composite Design (CCD) of the Experiment

Three important parameters identified following enzyme production from one factor at a time (OFAT) were further optimized using Central Composite Design (CCD) in Response Surface Methodology (RSM). A total of twenty (20) different experiments each from alkaliphilic *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 were randomly designed by the software and later conducted in our laboratory as predicted. The independent variables were pH (A), temperature (B) and ammonium sulphate concentration (C). The predicted and actual responses from the central composite experimental plan of *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 for both CMCase and FPase were summarized in **Table 6.6** and **6.7** respectively. An estimated endoglucanase and exoglucanase as a function of independent variables were obtained after ANOVA has provided a regression equations. Models precisions were normally determined by coefficient (R^2).

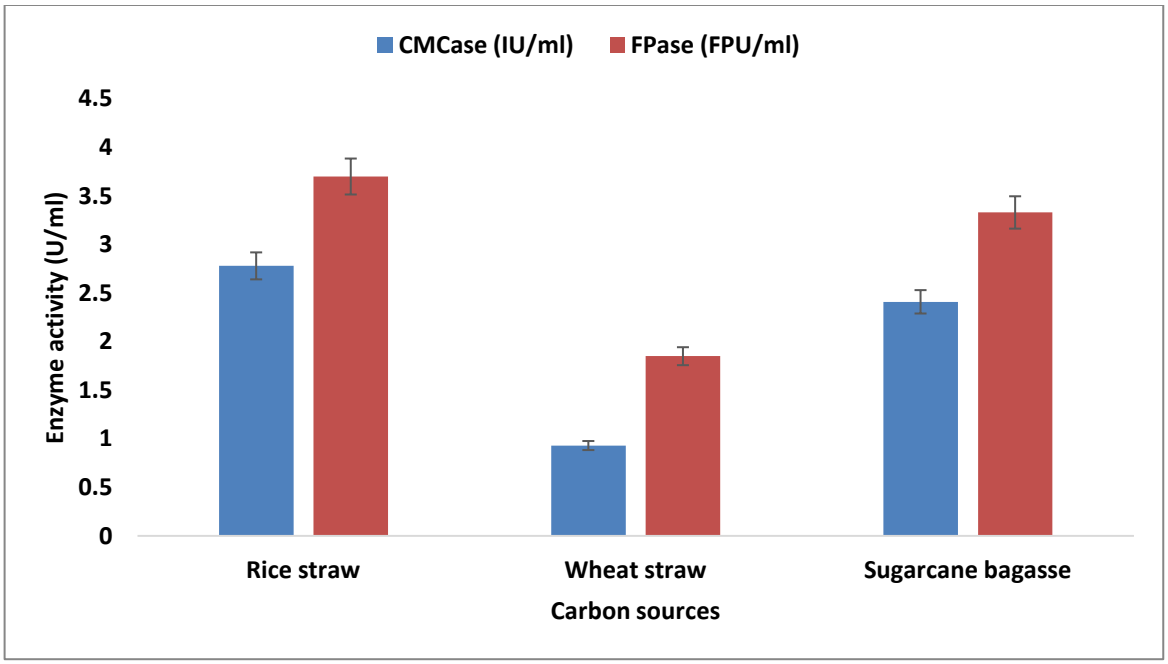


Fig. 6.22.0: Effect of different carbon sources on the production of CMCase and FPase from *Fusarium oxysporum* VSTPDKF2. Error bars represent mean±standard deviation

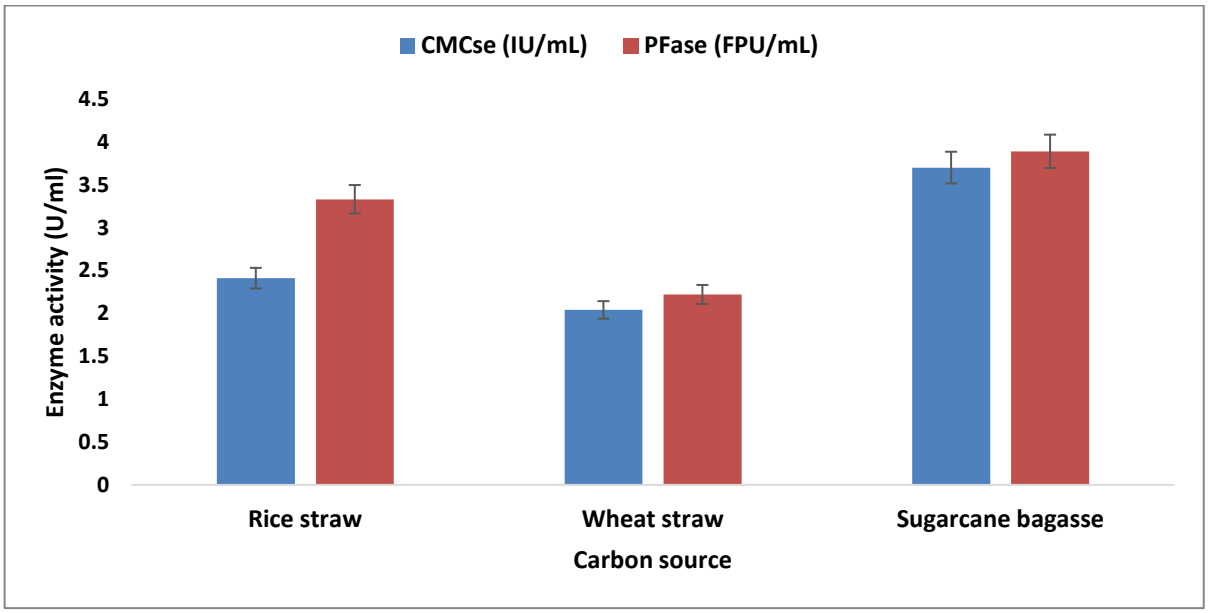


Fig. 6.23.0: Effect of different carbon sources on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *M. circinelloides* WSSDBS2F1. Error bars represent mean±standard deviation

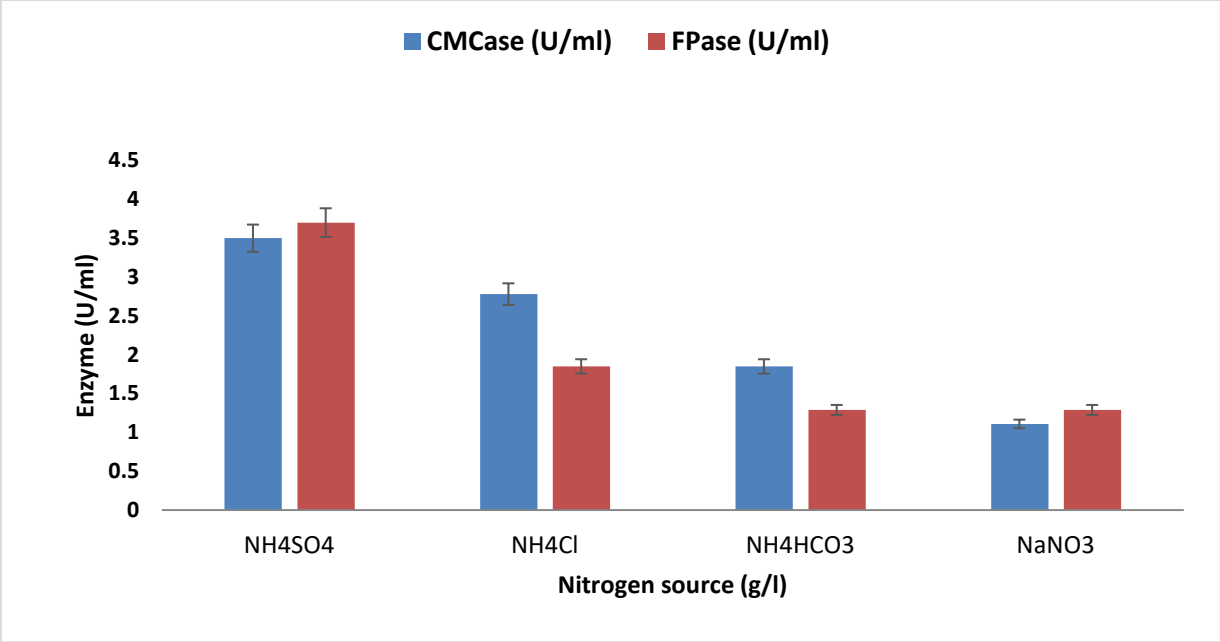


Fig. 6.24.0: Effect of different nitrogen sources on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *Fusarium oxysporum* VSTPDKF2. Error bars represent mean±standard deviation

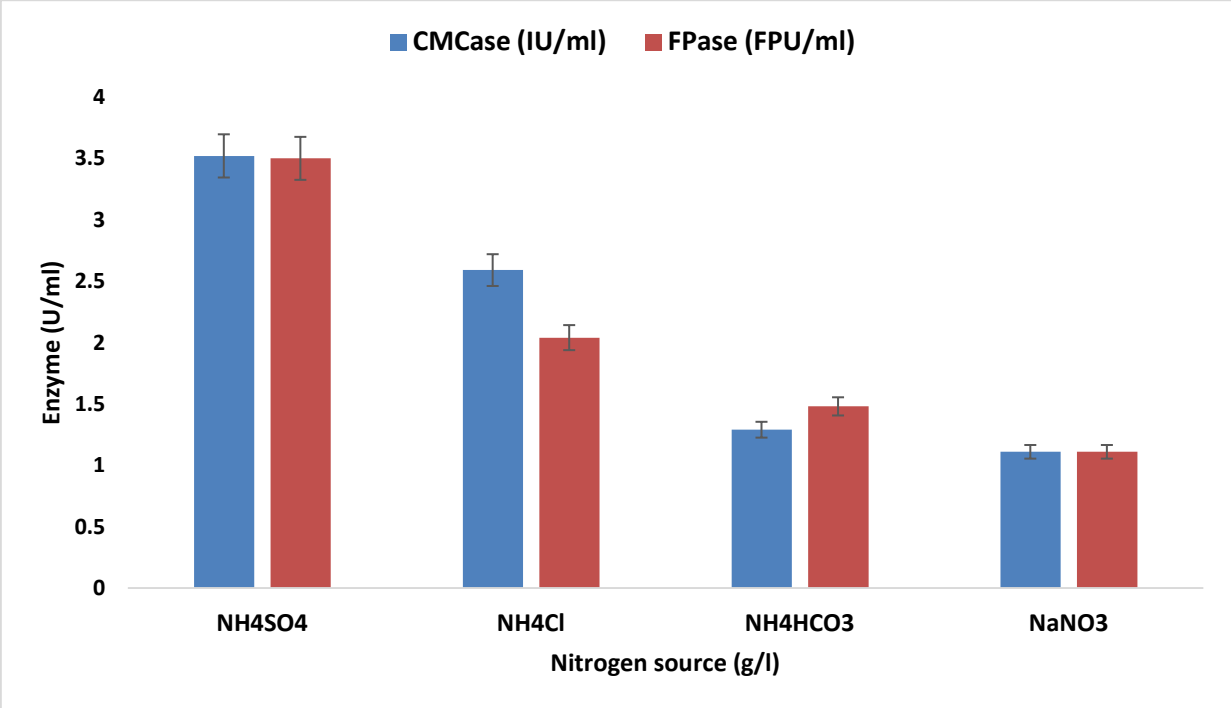


Fig. 6.25.0: Effect of different nitrogen source on the production of CMCase and FPase from *M. circinelloides* WSSDBS2F1. Error bars represent mean±standard deviation

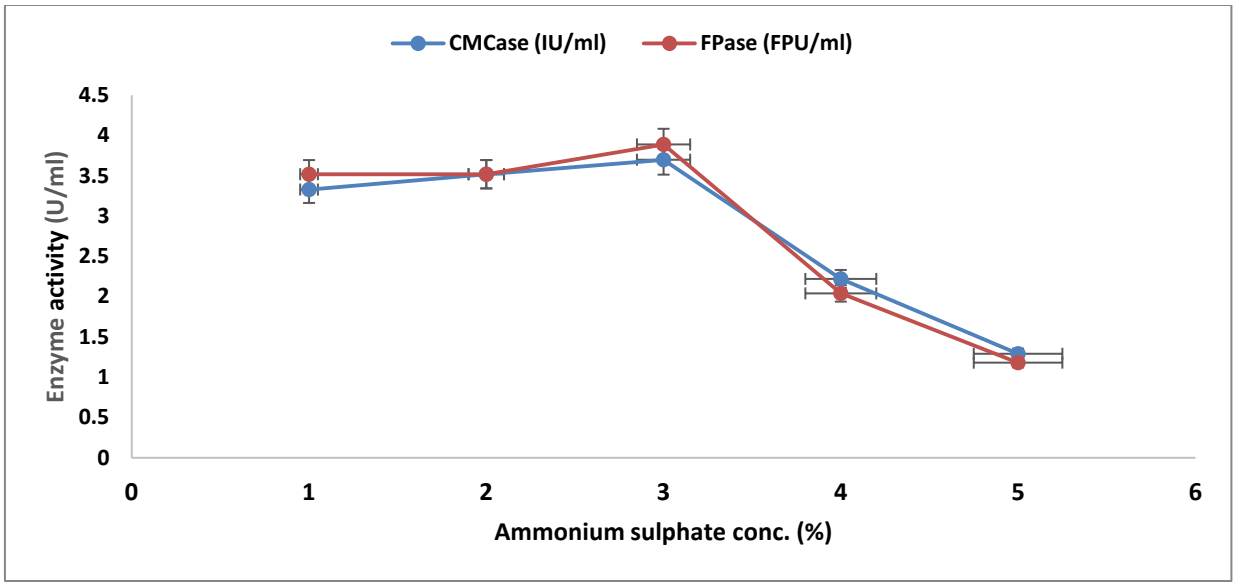


Fig. 6.26.0: Effect of ammonium sulphate concentration on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *Fusarium oxysporum* VSTPDKF2. Error bars represent mean±standard deviation

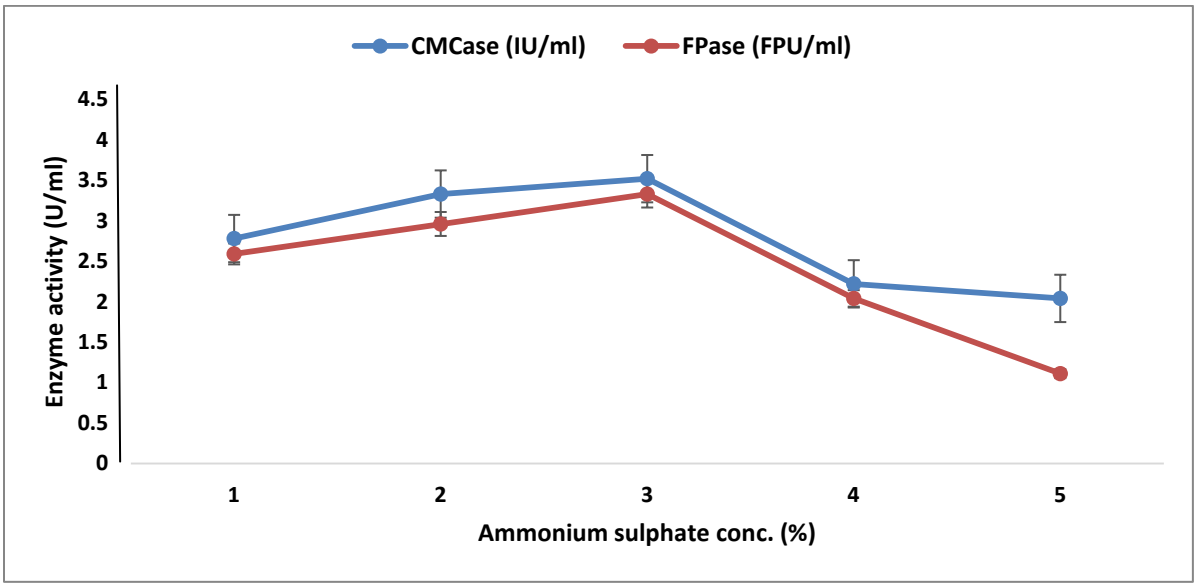


Fig. 6.27.0: Effect of ammonium sulphate concentration on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *Mucor circinelloides* WSSDBS2F1. Error bars represent mean±standard deviation

Its values always range between 0 to 1, where the order of magnitude suggest goodness of the model (Borugadda and Goud, 2015). The R^2 values of CMCase and FPase from *F. oxysporum* VSTPDKF2 were found to be 0.9933 and 0.9894 respectively while R^2 values of CMCase and FPase enzymes produced from *M. circinelloides* WSSDBS2F1 are 0.9852 and 0.9899 respectively. These R^2 values are close to 1 which signifies that the 99.33% and 98.94% behavior of the models from *F. oxysporum* VSTPDKF2 as well as 98.52% and 98.99% behavior from *M. circinelloides* WSSDBS2F1 can be interpreted for alkaline cellulase enzyme production while only 0.67% and 1.06%, as well as 1.48% and 1.01% full variance respectively cannot be explained by the models. According to Yusuf *et al.,* (2016), for high accuracy and ability of polynomial model to be good, R^2 value must be close to 1. A similar R^2 value of 0.9873 and 0.8770 for CMCase and FPase respectively was reported by Kumar *et al.*, (2018). The Adjusted R^2 from this model was 0.9873 and 0.9798 as well as 0.9721 and 0.9808 for *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 respectively. This is an indication of a good relationship between actual and predicted values. From the results obtained, predicted R^2 values for CMCase (0.9710 and 0.9388) and FPase (0.9300 and 0.9279) agreed with Adjusted R^2 values of 0.9873 and 0.9798 respectively. Hence, the model provides clarity on the relationship between response and independence variables. Adequate precisions of the model measured the signal to noise ratio for CMCase and FPase from *F. oxysporum* VSTPDKF2 (48.790 and 39.373) and *M. circinelloides* WSSDBS2F1 (31.58 and 36.622) respectively indicated an adequate signal while the result shows that the model is significant. This result agreed with that of Sharma *et al.,* (2009) which reported an adequate precision values of 17.4 and 14.4 respectively when optimizing nutrients supplements on the removal of Cr (VI) by *Aspergillus lentulus* AML05.

Significant of the models are generally measured based on P-value of F-value (prob > F). The higher the F-value and corresponding lower prob > F value, the better the importance of the corresponding coefficients (R^2) (Hassanzadeh-Tabrizi and Taheri-Nassaj 2011). For maximum alkaline cellulase enzyme production, second-order response surface models in the form of ANOVA for CMCase and FPase from *F. oxysporum* VSTPDKF2 were summarized in **Table 6.8** while CMCase and FPase produced from alkaline *M. circinelloides* WSSDBS2F1 were summarized in **Table 6.9**.

Table 6.6.0: Experimental design having coded levels of variables used in CCD with experimental and predicted value for CMCase and FPase activity from *F. oxysporum* VSTPDKF2

Standard Order	Run	Factor1 pH	Factor 2 Temperature (^{O}C)	Factor3 NH_4SO_4 (%)	CMCase (IU/ml)		FPase (FPU/ml)	
					Actual	Predicted	Actual	Predicted
1	16	0	0	0	3.89	3.91	4.26	4.26
2	3	1	0	0	1.11	1.16	0.74	0.89
3	11	1	-1	1	0.37	0.33	0.37	0.37
4	4	-1	-1	-1	0.19	0.19	0.19	0.16
5	7	0	0	0	2.96	3.00	3.15	3.23
6	20	-1	1	-1	0.56	0.63	0.37	0.41
7	5	0	0	0	0.00	-0.02	0.00	-0.11
8	6	0	0	1	0.19	0.21	0.19	0.23
9	15	1	-1	-1	1.11	1.11	1.29	1.31
10	12	1	1	-1	0.00	-0.14	0.00	-0.02
11	17	-1	1	1	2.77	2.59	2.77	2.50
12	8	0	0	0	0.56	0.60	0.37	0.46
13	13	-1	-1	1	0.74	0.71	0.93	0.80
14	9	0	1	0	0.37	0.26	0.37	0.32
15	10	-1	0	0	0.74	0.70	0.56	0.71
16	14	0	0	-1	0.74	0.70	0.74	0.71
17	19	0	-1	0	0.74	0.70	0.74	0.71
18	1	1	1	1	0.56	0.70	0.74	0.71
19	18	0	0	0	0.56	0.70	0.56	0.71
20	2	0	0	0	0.56	0.70	0.56	0.71

Table 6.7: Experimental design having coded levels of variables used in Central Composite Design (CCD) with experimental and predicted value for CMCase and FPase activity from *M. circinelloides* WSSDBS2F1

Standard Order	Run	Factor1 pH	Factor 2 Temperature (^{O}C)	Factor3 NH_4SO_4 (%)	CMCase (IU/ml)		FPase (FPU/ml)	
					Actual	Predicted	Actual	Predicted
1	4	-1	-1	-1	3.33	3.41	3.64	3.64
2	9	1	-1	-1	0.19	0.25	0.19	0.13
3	6	-1	1	-1	0.37	0.33	0.56	0.52
4	10	1	1	-1	0.19	0.23	0.19	0.24
5	13	-1	-1	1	4.26	4.30	4.26	4.27
6	3	1	-1	1	0.37	0.48	0.37	0.48
7	11	-1	1	1	0.74	0.76	0.56	0.68
8	18	1	1	1	0.00	1.023E-003	0.19	0.13
9	15	-1	0	0	2.03	1.94	2.41	2.20
10	2	1	1	-1	0.19	-0.021	0.19	0.16
11	17	0	-1	0	2.41	2.13	2.22	2.04
12	14	0	1	0	0.37	0.35	0.37	0.30
13	16	-1	-1	1	0.37	0.24	0.37	0.30
14	8	0	0	1	0.74	0.57	0.74	0.56
15	7	-1	0	0	0.56	0.69	0.56	0.76
16	20	0	0	0	0.56	0.69	0.74	0.76
17	12	0	0	0	0.37	0.69	0.74	0.76
18	1	0	0	0	0.56	0.69	0.56	0.76
19	19	0	0	0	0.74	0.69	0.74	0.76
20	5	0	0	0	0.74	0.69	0.74	0.76

Table 6.8.0: Central Composite Design (CCD) optimization result summary of ANOVA for CMCase and FPase from *Fusarium oxysporum* VSTPDKF2

Parameters	**Result**		**Remark**
	CMCase	**FPase**	
F- value	165.31	103.28	
Prob > F	<0.0001	<0.0001	Significant
R^2 value	0.9933	0.9894	
Adjusted R^2	0.9873	0.9798	
Predicted R^2	0.9710	0.9300	
Adequate precision	48.790	39.373	Adequate signal to noise ratio
Lack of fit F value	1.83	4.29	
Lack of fit prob > F	0.2613	0.0679	Not significant

Fig. 6.9 Central Composite Design (CCD) optimization result summary of ANOVA for CMCase and FPase from *M. circinelloides* WSSDBS2F1

Parameters	**Result**		**Remark**
	CMCase	**FPase**	
F- value	74.56	109.01	
Prob > F	<0.0001	<0.0001	Significant
R^2 value	0.9853	0.9899	
Adjusted R^2	0.9721	0.9808	
Predicted R^2	0.9388	0.9279	
Adequate precision	31.958	36.622	Adequate signal to noise ratio
Lack of fit F value	2.80	4.93	
Lack of fit prob > F	0.1414	0.0524	Not significant

These results indicated that high models *F*-value for alkaline CMCase and FPase from *F. oxysporum* VSTPDKF2 are 165.31 and 103.28 with their respective small prob >F values (P-value) of <0.0001 signify that the models were significant. Meanwhile, CMCase and FPase produced from alkaline *M. circinelloides* WSSDBS2F1 recorded an *F*-value of 74.56 and 109.01 respectively with their corresponding prob >F values (P-value) of <0.0001 indicated that that the model is significant. This means the probability where the F value model could happen due to noise was 0.01%. To ensure the importance of each coefficient, P-values are adopted as tools. The prob>F<0.05 values showed that the models were significant. This means that A, B, C, A^2, B^2, C^2, AB, AC as well as BC are the significant model terms. The lack of fit *F*-value of the models for both CMCase and FPase from *F. oxysporum* VSTPDKF2 are 1.83 and 4.29 with their corresponding P-values were 0.2613 and 0.0679 respectively **(Table 6.10 and 6.11).** The lacks of fit F-value of the models for CMCase and FPase from alkaline *M. circinelloides* WSSDBS2F1 are 2.80 and 4.93 with their corresponding lack of fit P-value as 0.1414 and 0.0524 respectively indicated that lack of fits was not significant and that models are very accurate without any noise **(Table 6.12 and 6.13)**. Borugadda and Goud (2015) reported that a lack of fit must be estimated to examined analysis of variance (ANOVA) on each model coefficient and ensure an in-depth model fit. Manogaran *et al.,* (2018) and Ibrahim *et al.,* (2015) reported a non-significant lack of fits by describing it as an excellent fit. Based on the result obtained, there was a strong relationship between actual and predicted model values for both CMCase and FPase as depicted in equation 6.1 and 6.2 for CMCase as well as 6.3 and 6.4 for FPase which described actual and coded factors respectively.

CMCase = (0.70) + (-0.63xA) + (1.00xB) + (0.22xC) + (0.21xA^2) + (0.90xB^2) + (0.21xC^2) + (0.65xAB) + (0.094xAC) + (0.14xBC) **(Equation 6.1)**

CMCase = (19.07785) + (-0.51475*A) + (-0.58746*B) + (-0.16350*C) + (-0.033600xA^2) + (4.00000E-003*B^2) + (-0.052500*C^2) + (0.017300*AB) + (0.018750*AC) + (4.62500E-003*BC)

(Equatin 6.2)

FPase = (0.71) + (-0.76xA) + (-1.02xB) + (-0.24xC) + (-0.15xA^2) + (0.77xB^2) + (-0.15xC^2) + (0.79xAB) + (0.14xAC) + (0.14xBC) **(Equation 6.3)**

FPase = (20.92858) + (-0.91753xA) + (-0.5693xB) + (-0.34291xC) + (-0.024436xA^2) (-0.56937xB) + (-0.34291xC) + (-0.024436xA^2) + (3.43232E-003xB^2)+ (-0.036932xC^2) + (0.021033xAB) + (0.027750xAC) + (4.62500E-003xBC) **(Equation 6.4)**

The 3D response surface as well as their contour plots in central composite design (CCD) shows an interaction between the two different factors while keeping other factors constant **(Fig. 6.28, 6.29, 6.30 and 6.31)**. This visualization helps to understand the interaction between two factors and pinpoint the optimum level of each parameter for maximal response (de Lima *et al*., 2010). The interaction between ammonium sulphate concentration and enzyme production showed an important effect. Increases in ammonium sulphate concentration will increases enzyme production from 1 to 3% and where subsequent increase showed a decrease in enzyme production. The optimum enzyme production was indicated at 3% ammonium sulphate and pH 8.5. An excellent correlation was also observed between pH and temperature as well as temperature and ammonium sulphate concentration.

6.9 Validation of statistical experiments

The accurate prediction and model's fitness evaluation of each variable investigated requires validation of the model from both a graphical and numeric approach. Interpretation of result in such matter as graphic and numeric have a key role in variable combination as well as concluding the effect of each variable. To examine the significance of the facts, simple statistical and mathematical tests such as F test, P-value and ANOVA were used in validating the experimental design. Analysis of variance (ANOVA) was used which is the best reliable statistical test to examine the goodness of the model fitness by comparing changes based on the treatment and random inherent errors in the determination of analyzed responses (Aanchal *et al.*, 2016). Based on the RSM-CCD results, the optimum conditions for enzyme production were 8.5 initial pH, 45^{O}C temperature and 3% ammonium sulphate concentration, while incubation time was kept constant (8 days). *F. oxysporum* VSTPDKF2, RSM experiments reported an actual enzyme production of 3.91 IU/ml and 4.26 FPU/ml for CMCase and FPase respectively. Result from *M. circinelloides* WSSDBS2F1 both CMCase and FPase reported an actual enzyme production of 4.26 IU/ml. These experiments were conducted based on the different predicted experiments from three factors identified as pH, temperature and NH_4SO_4 concentration in order

to validate the results previously conducted with CCD. Comparison between OFAT and RSM from *F. oxysporum* VSTPDKF2 revealed an increased enzyme production of 0.39 IU/ml (CMCase) and 0.19 FPU/ml (FPase) using RSM as compared to that of OFAT **(Table 6.14)**. Similarly, *M. circinelloides* WSSDBS2F1 RSM result revealed an increased in enzyme activity of 0.74 IU/ml and 0.56 FPU/ml for CMCase and FPase respectively as compared to OFAT **(Table 5.15)**. This indicated that RSM can be used to increase cellulase enzyme activity as compared to OFAT and other conventional methods of enzyme productions.

6.10.0 Evaluation of endoglucanase enzyme deinking potentials

The application of enzymes has been suggested as an environmentally friendly alternative way of replacing conventional deinking in the recycling waste paper (Ibarra et al., 2012). The present study was aimed at isolating alkaliphilic fungi by producing alkaline cellulase enzyme from the soil and some industrial effluents of Kapurthala and Amritsar district Punjab with potential evaluation on the removal of ink from waste paper. In this research, alkaline endoglucanase (CMCase) and exoglucanase (FPase) enzyme produced from *F. oxsporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 were selected and optimized using design expert software. An A4 paper was printed with word 'deinking' for approximately 640 words using xerox printer was used as waste paper. The flotation was conducted using a simple instrument constructed in our laboratory (see appendix). The optimized endoglucanase was later evaluated for deinking capability together with chemical deinking and a control. The chemicals used in replacing the enzyme were with 2% Na_2SiO_3, 2% NaOH and 1% H_2O_2 while 0.1% tween 80 acts as surfactants and 0.1% $CaCl_2$ was used as a flotation aid. After enzymatic, chemical and control deinking process as well as flotation, the resultant pulp was prepared according to TAPPI standard and sent to the Central Pulp & Paper Research Institute (CPPRI) Saharanpur, 247001 (U.P.) India for the testing of pulp brightness, tensile strength, bursting strength and tearing strength.

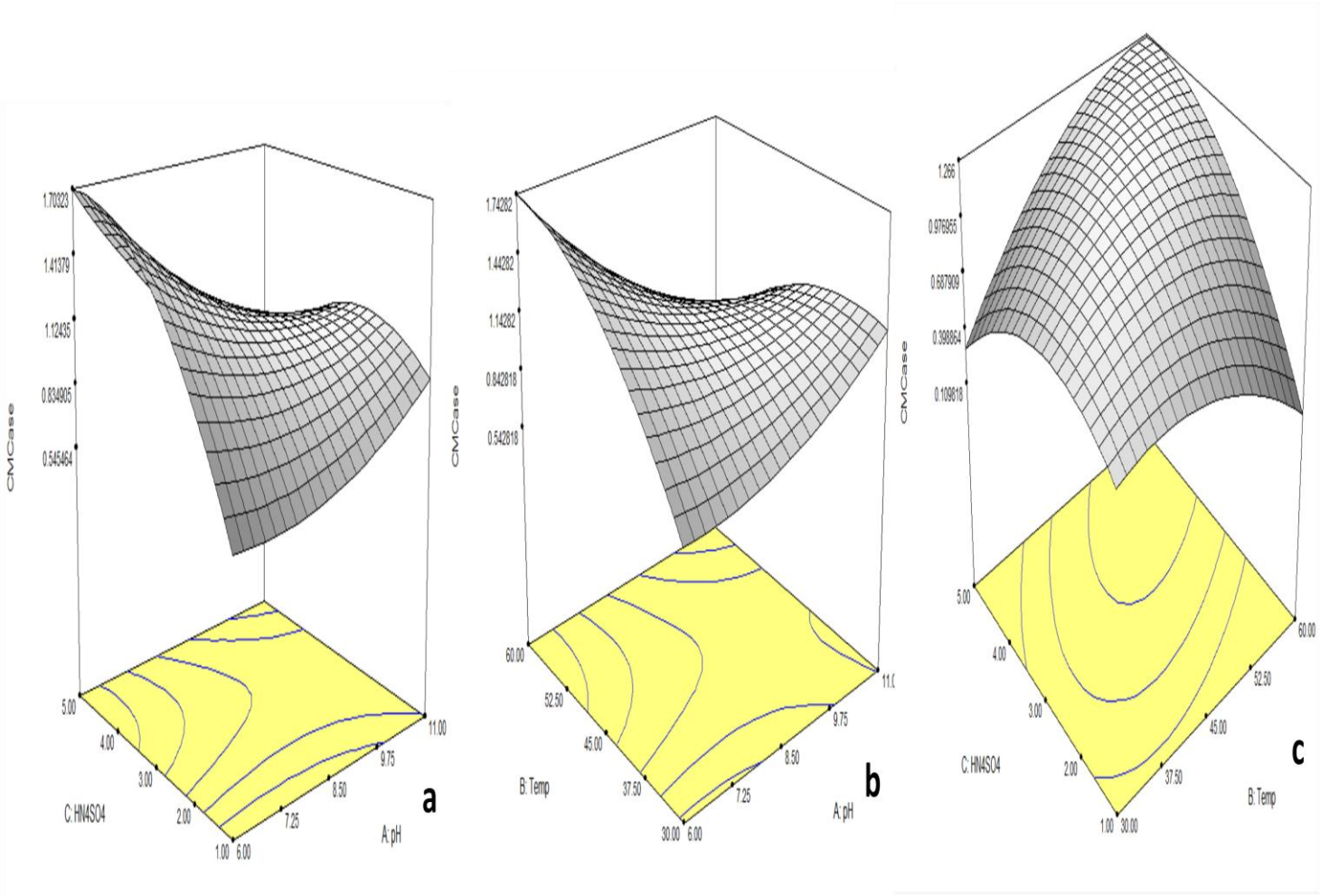


Fig. 6.28.0: A three 3D response surface plots for the optimization of endoglucanase from *Fusarium oxysporum* VSTPDKF2 having interactive effects of the three different parameters

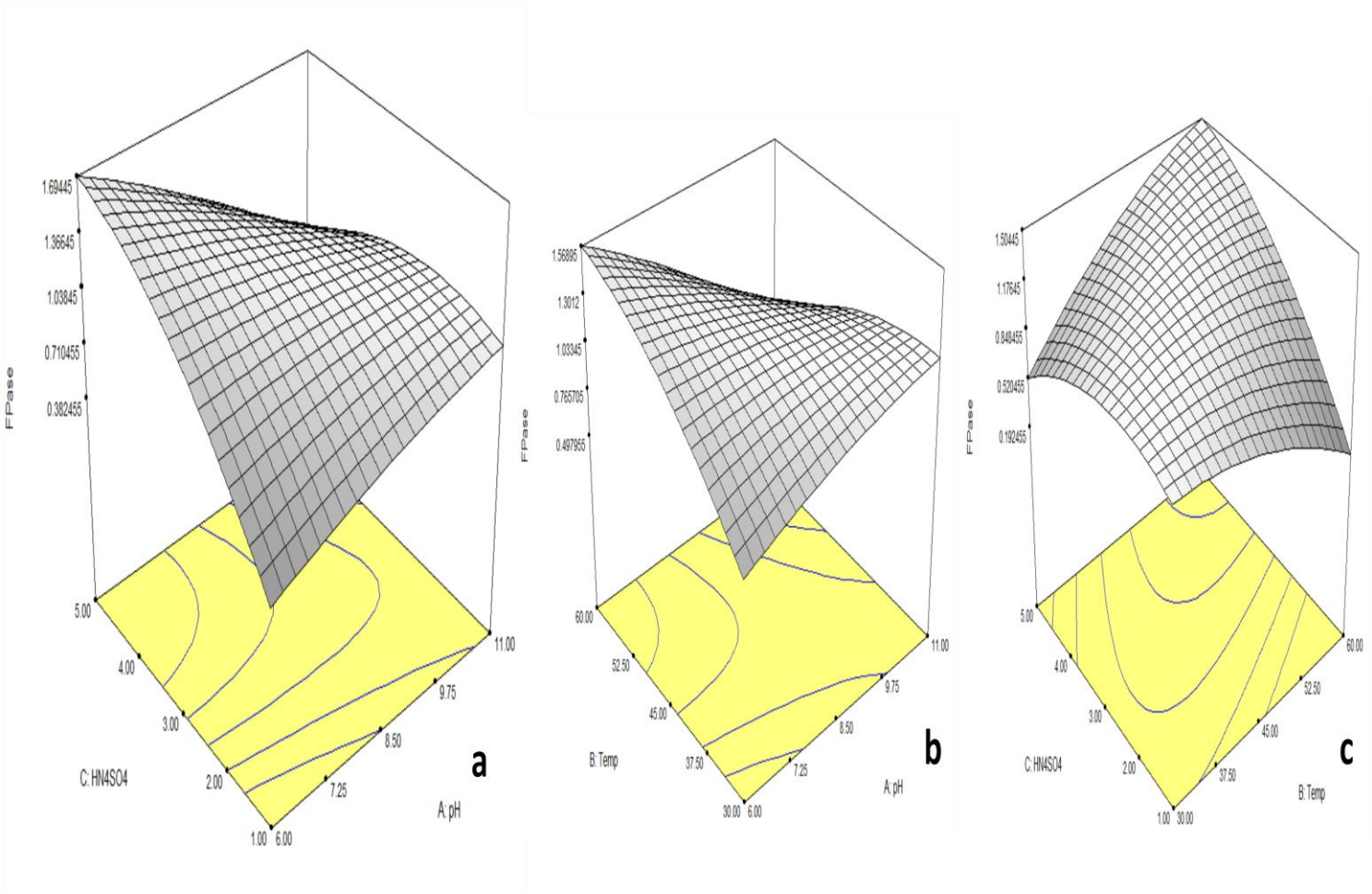


Fig. 6.29.0: A three 3D response surface plots for the optimization of exoglucanase from *Fusarium oxysporum* VSTPDKF2 having interactive effects of the three different parameters

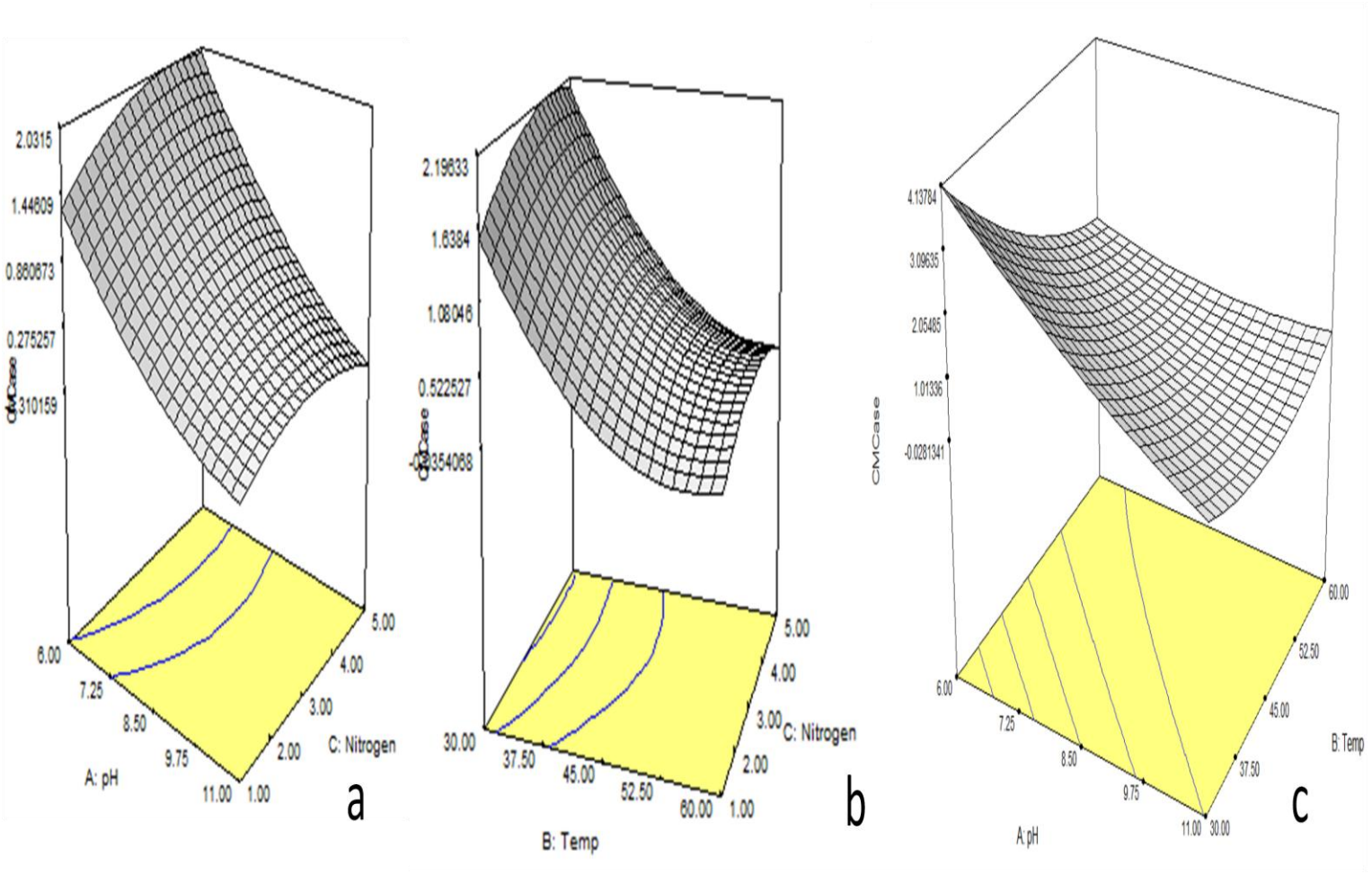


Fig. 6.30.0: A three 3D response surface plots for the optimization of endoglucanase from *Mucor circillinoides* WSSDBS2F1 having interactive effects of the three different parameters

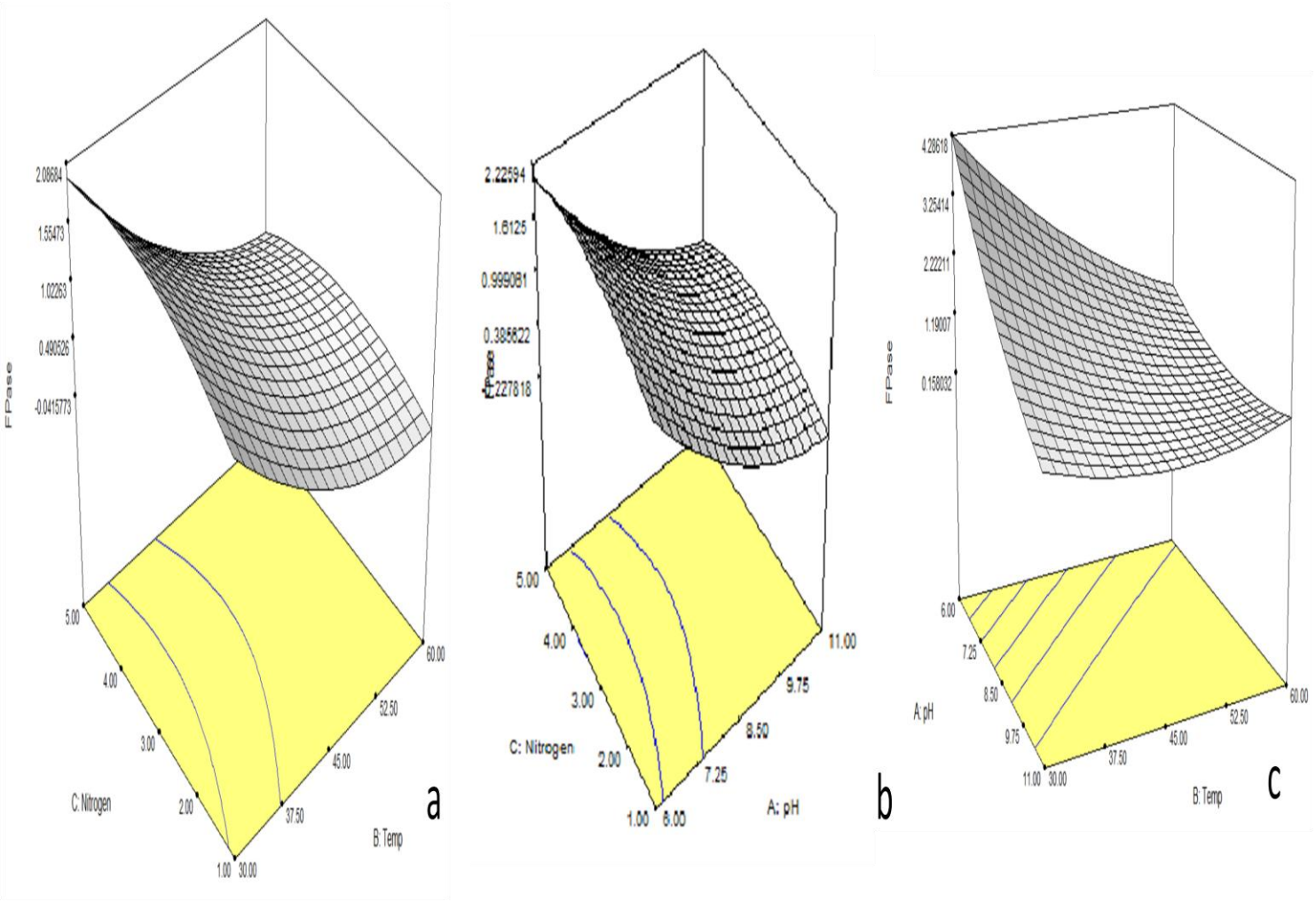


Fig. 6.31.0: A three 3D response surface plots for the optimization of exoglucanase from *Mucor circillinoides* WSSDBS2F1 having interactive effects of the three different parameters

Table 6.10.0: Analysis of variance (ANOVA) for endoglucanase (CMCase) from *Fusarium oxysporum* VSTPDKF2

Source	Sum of square	DF	Mean square	*F* value	Prob>F value	Remark
Model	20.48	9	2.28	165.31	< 0.0001	Significant
A = pH	3.94	1	3.94	286.57	< 0.0001	-
B = temperature	9.96	1	9.96	723.72	< 0.0001	-
C = NH_4SO_4	0.49	1	0.49	35.81	0.0001	-
A^2	0.12	1	0.12	8.81	0.0141	-
B^2	2.23	1	2.23	161.86	< 0.0001	-
C^2	0.12	1	0.12	8.81	0.0141	-
AB	3.37	1	3.37	244.66	< 0.0001	-
AC	0.070	1	0.070	5.11	0.0473	-
BC	0.15	1	0.15	11.19	0.0074	-
Residual	0.14	10	0.014	-	-	-
Lack of fit	0.089	5	0.018	1.83	0.2613	Not significant
Pure error	0.049	5	9.720E-003	-	-	-
Cor Total	20.61	19	-	-	-	-

Table 6.11.0: Analysis of variance (ANOVA) for exoglucanase (FPase) from *Fusarium oxysporum* VSTPDKF2

Source	Sum of square	DF	Mean square	*F* value	Prob > F value	Remark
Model	23.91	9	2.66	103.28	< 0.0001	Significant
A = Ph	5.75	1	5.75	223.41	< 0.0001	-
B = Temperature	10.34	1	10.34	402.17	< 0.0001	-
C = NH_4SO_4	0.58	1	0.58	22.58	0.0008	-
A^2	0.064	1	0.064	2.49	0.1453	-
B^2	1.64	1	1.64	63.77	< 0.0001	-
C^2	0.060	1	0.060	2.33	0.1576	-
AB	4.98	1	4.98	193.52	< 0.0001	-
AC	0.15	1	0.15	5.99	0.0344	-
BC	0.15	1	0.15	5.99	0.0344	-
Residual	0.26	10	0.026	-	-	-
Lack of fit	0.21	5	0.042	4.29	0.0679	Not significant
Pure error	0.049	5	9.720E-003	-	-	-
C or total	24.16	19	-	-	-	-

Table 6.12.0: Analysis of variance (ANOVA) surface quadratic model for endoglucanase (CMCase) produced from alkaline *M. circinelloides* WSSDBS2F1

Source	**Sum of square**	**DF**	**Mean square**	***F* value**	**Prob>F value**	**Remark**
Model	25.12	9	2.79	109.01	< 0.0001	Significant
A = pH	10.36	1	10.36	404.68	< 0.0001	-
B = temperature	7.55	1	7.55	294.89	< 0.0001	-
C = NH_4SO_4	0.17	1	0.17	6.50	0.0289	-
A^2	0.48	1	0.48	18.62	0.0015	-
B^2	0.47	1	0.47	18.17	0.0017	-
C^2	0.30	1	0.30	11.60	0.0067	-
AB	5.25	1	5.25	204.96	< 0.0001	-
AC	0.039	1	0.039	1.53	0.2443	-
BC	0.11	1	0.11	4.13	0.0695	-
Residual	0.26	10	0.026	-	-	-
Lack of fit	0.21	5	0.043	4.93	0.0524	Not significant
Pure error	0.043	5	8.640E-003	-	-	-
C or Total	25.38	19	-	-	-	-

Table 6.13.0: Analysis of variance (ANOVA) surface quadratic model for exoglucanase (FPase) produced from alkaline *M. circinelloides* WSSDBS2F1

Source	Sum of square	DF	Mean square	*F* value	Prob>F value	Remark
Model	24.50	9	2.72	74.56	< 0.0001	Significant
A = pH	9.58	1	9.58	269.48	< 0.0001	-
B = temperature	7.90	1	7.90	216.44	< 0.0001	-
C = NH_4SO_4	0.28	1	0.28	7.55	0.0206	-
A^2	0.20	1	0.20	5.42	0.0422	-
B^2	0.83	1	0.83	22.63	0.0008	-
C^2	0.23	1	0.23	6.20	0.0320	-
AB	4.67	1	4.67	127.80	0.0001	-
AC	0.21	1	0.21	5.87	0.0358	-
BC	0.11	1	0.11	2.96	0.1160	-
Residual	0.37	10	0.037	-	-	-
Lack of fit	0.27	5	0.054	2.80	0.1414	Not significant
Pure error	0.096	5	0.019	-		-
C or Total	24.87	19	-	-	-	-

Table 6.14.0: Validation of optimum conditions and results obtained between OFAT and RSM for the optimization of CMCase and FPase from *F. oxysporum* VSTPDKF2

Factors	**CMCase (IU/ml)**		**FPase (FPU/ml)**	
	OFAT	**RSM**	**OFAT**	**RSM**
pH	6	8.5	6	8.5
Temperature (^{O}C)	30	45	30	45
NH_4SO_4 (%)	3	3	3	3
Enzyme activity	3.52	3.89	4.07	4.26
Enzyme increase	-	**0.37**	**-**	**0.19**

Table 6.15.0: Validation of optimum conditions and results obtained between OFAT and RSM for the optimization of CMCase and FPase from *M. circinelloides* WSSDBS2F1

Factors	**CMCase (IU/ml)**		**FPase (FPU/ml)**	
	OFAT	**RSM**	**OFAT**	**RSM**
pH	6	8.5	6	8.5
Temperature (^{O}C)	30	45	30	45
NH_4SO_4 (%)	3	3	3	3
Enzyme activity	3.52	4.26	3.70	4.26
Enzyme increase	-	**0.74**	**-**	**0.56**

The result in **Table 6.16** shows the optical and mechanical properties of the prepared hand sheet which includes percentage brightness, tensile strength, bursting strength and tearing strength after enzymatic treatment with crude alkaline endoglucanase isolated from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 as well as chemical deinking and control. The percentage brightness of 81.99% and 81.63 were recorded for *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 as compared to 81.51% and 79.63% for chemical treatment and control respectively. The percentage brightness result following *F. oxysporum* VSTPDKF2 enzymatic treatment indicated an increase ISO brightness of 2.36% and 0.48% as compared to control and chemical respectively from the prepared hand sheet. Moreover, 1.00% and 0.12% improvement in ISO brightness were recorded following enzymatic treatment of CMCase from *M. circinelloides* WSSDBS2F1 **(Fig.6.32)**. Many papers have reported the deinking of different waste papers from many fungal species including *Aspergillus oryzae* MDU-4, *Ganoderma lucidum* MDU-7, *Trichoderma citrinoviride* MDU-1, *Trichoderma harzyianum* PPDDN10, *Mucor hiemalis Aspergillus sp, Aspergillus terreus* AN1, *Myceliophthora fergusii* T41 (Roushdy, 2015; Saini, *et al.,* 2020; Soni *et al.,* 2010; Pathak, *et al.,* 2014b). In most of these reported papers, the fungi were isolated at a pH within the acidic to neutral range. The mechanical properties of the hand sheet for alkaline CMCase isolated from *F. oxysporum* VSTPDKF2 includes 1080N/m tensile strength, 59KPa burst strength and 288mN tear index while that of *M. circinelloides* WSSDBS2F1 are 1250N/m tensile strength, 67KPa burst strength and 352mN tear index. The mechanical properties of control and chemical deinking experiment include tensile strength (1070 and 953N/m), bursting strength (56 and 58KPa) as well as tear index 285 and 279mN respectively. Many papers reported the application of commercial cellulase either singly or in combination with other enzymes for deinking of different waste papers (Pathak *et al.,* 2011; Pathak *et al.,* 2014a; Ibarra *et al.,* 2012; Tsatsis *et al.,* 2017; Singh *et al*. 2012; Zhang *et al.,* 2008; Lee, *et al.,* 2013). However, these commercial enzymes are expensive and as such a low-cost enzyme for the deinking purpose from fungi using abundant lignocellulosic wastes that are available in Punjab such as rice straw, wheat straw and sugarcane bagasse as carbon sources are of paramount importance.

Over the past few years, efforts were made by many researchers in the production of cellulase enzymes by fungi using cheaply available lignocellulosic wastes (Roushdy, 2015; Soni *et al*., 2008). This process was found to be effective in the removal of ink from waste paper and

improvement of ISO brightness as well as an increase in the mechanical strength of the paper. In another development, combination enzymatic and chemicals in this regards was also found to be more effective as compared to a single application of either enzymatic or conventional method. Combination of chemical and enzyme for deinking was reported to improve optical and mechanical properties of deinking paper as well as properties of pulp and generated wastewater effluent (Lee *et al.*, 2011; Pala *et al*., 2004). Most of these researches were conducted from acidic to neutral pH since fungi grow best at acidic conditions. Few papers are found to report waste paper deinking using alkaliphilic fungal cellulase with improvement in brightness and mechanical strength. Alkaline extracellular endoglucanase was produced from two *Basidiomycetes* that grow at pH 8.0. In related work, Lee and Eom (1999) reported the effectiveness of alkaline endoglucanase isolated from *Coprinus cinereus* 2249 for the deinking old newsprint. They concluded that the application of the enzyme at pH 9 can reduce the use of chemicals as well as a decrease in environmental hazard. Hand sheet made from prepared pulp was found to have an increase in optical as well as mechanical strength when two alkalotolerent *Fusarium sp* was used for the removal of ink from mixed office waste paper by Vyas and Lachke (2003). In this research, we reported production of alkaline cellulase enzyme capable of removing ink from waste paper from two novel alkalothermophilic fungi.

Most of the previously reported papers on enzyme deinking also reported an increase in tensile, tearing and bursting strength. Tensile strength or tensile index refers to the maximum tensile stress developed in a test specimen before rupture. It normally indicated the amount of force needed to tear the paper when pulled at opposite ends and in opposite directions. Enzymatic deinking using CMCase produced from *F. oxysporu*m VSTPDKF2 and *M. circinelloides* WSSDBS2F1 improved the tensile strength of the printed waste paper by 10N/m and 127N/m as well as 180N/m and 253N/m respectively when compared to control and chemical deinking process **(Fig. 6.33)**. In terms of bursting strength which is the hydrolytic pressure needed to breakdown material when pressure is increase at a constant rate, enzymatic deinking of the enzyme produced by these isolated fungi increase the bursting strength of the deink hand sheet by 3KPa and 1KPa from *F. oxysporu*m VSTPDKF2 and also 11KPa and 8KPa from *M. circinelloides* WSSDBS2F1 when compared to control and chemical deinking respectively. The third mechanical property of the prepared hand sheet tested was tearing strength as shown in **Fig 6.34.0**.

The result indicated an improvement of 3mN and 9mN, as well as 11mN and 8mN, tearing strength following enzymatic deinking alkaline CMCase isolated from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 respectively as compared to control and chemical deinking process respectively **(Fig. 6.35)**. Tearing strength is how a paper can withstand an applied tearing force normally measured in milliNewtons (mN). Majority of the papers previously reported for improvement of paper brightness also report either an increase or decrease of mechanical strength of the deink waste paper. Pathak et al., (2011) reported an increase of 15.3% and 21.9% for burst and tensile index respectively when using chemicals and commercial cellulase for deinking of photocopier paper. An increase of 1-14% tensile strength, 1.2-3.8% burst index, as well as decrease of 0.1-9.6% tear index as compared to chemical deinking process, was reported by Lee, *et al*., (2011) when old newsprint and mixed office waste paper are applied for the determination of deinking properties. Other papers also reported an increase as well as decrease of mechanical properties of various deinks waste papers (Lee *et al.*, 2013).

To confirm the influence of these enzymes for potential deinking ability, the prepared pulp was subjected to Scanning Electron Microscope (SEM) and Fourier Transformed Infrared Spectroscopy (FT RS). It is observed that enzymatic action usually resulted in internal fibrillation of fibre as well as some degree of surface fibrillation in the fibre. Increased strength can be signed to internal as well as surface fibrillation of the fibre by enzymatic action **(Fig. 6.36a and b; 6.37a and b; 6.38a and b)** through Scanning Electron Microscope (SEM) which can be clearly seen as compared to chemically deink **(Fig. 6.36c; 6.37c and 6.38c)** and control untreated pulp **(Fig. 6.36d, 6.37d and 6.38d)**. SEM pulp analysis revealed some changes such as crack development, pores as well as peeling off fibre. An increase in this fibrillation was observed in the case of enzymatic deink pulp when compared to control or chemically deink pulp as also reported by Pathak *et al.*, (2011). This development was similar with our finding when pulp treated with alkaline endoglucanase produced from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 was observed under a scanning electron microscope at different magnifications as shown in **Figure 6.36, 6.37 and 6.38**. Fibre swelling, loosing and peeling off of fibre are visible on enzymatic deink pulp when compared with control and chemically deink pulp.

Table 6.16: Optical and mechanical properties of enzymatic, chemical and control hand sheet

S/No.	Test parameter	Unit	Control	Chemical	VSTPDKF2	WSSDBS2F1
1	**Brightness**	%	79.63	81.51	81.99	81.63
2	**Tensile strength**	N/m	1070	953	1080	1250
3	**Bursting strength**	KPa	56	58	59	67
4	**Tearing strength**	mN	285	279	288	352

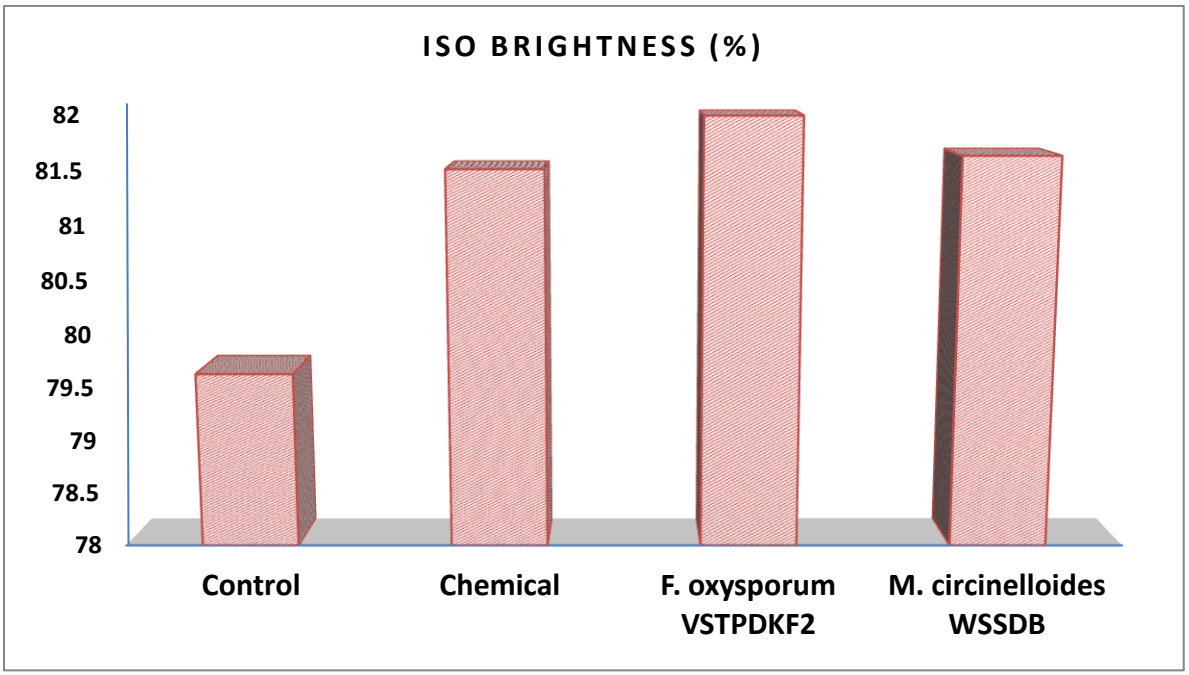


Fig. 6.32.0: Percentage brightness of enzymatic, chemical and control hand sheet

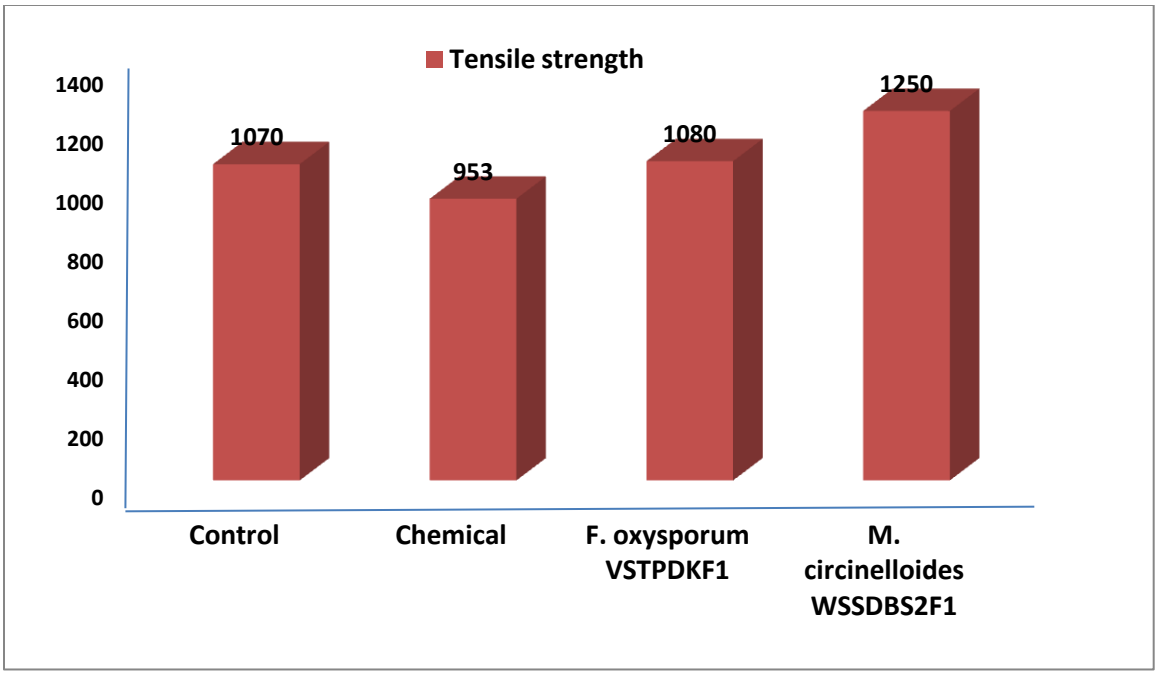


Fig. 6.33.0: Tensile strength of enzymatic, chemical and control pulp

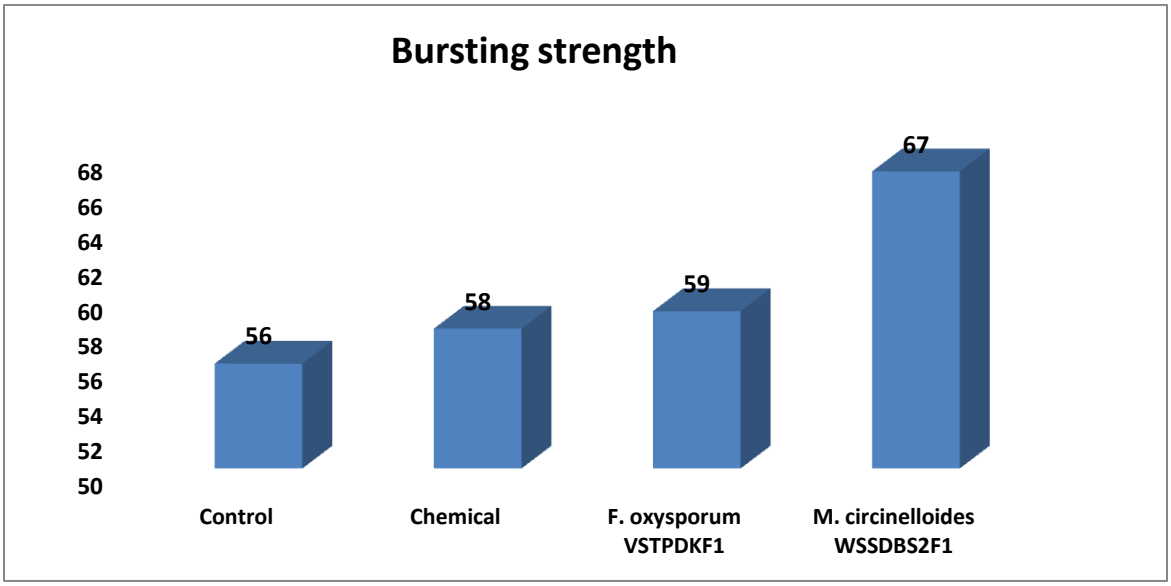


Fig. 6.34.0: Bursting strength of enzymatic, chemical and control pulp

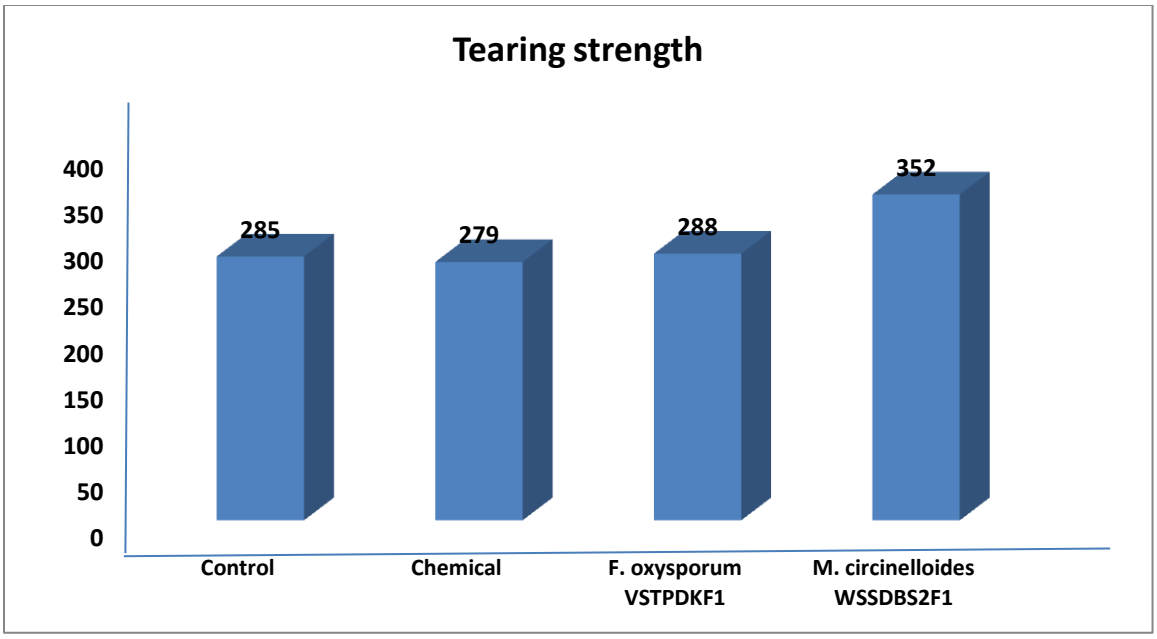


Fig. 6.35.0: Tearing strength of enzymatic, chemical and control pulp

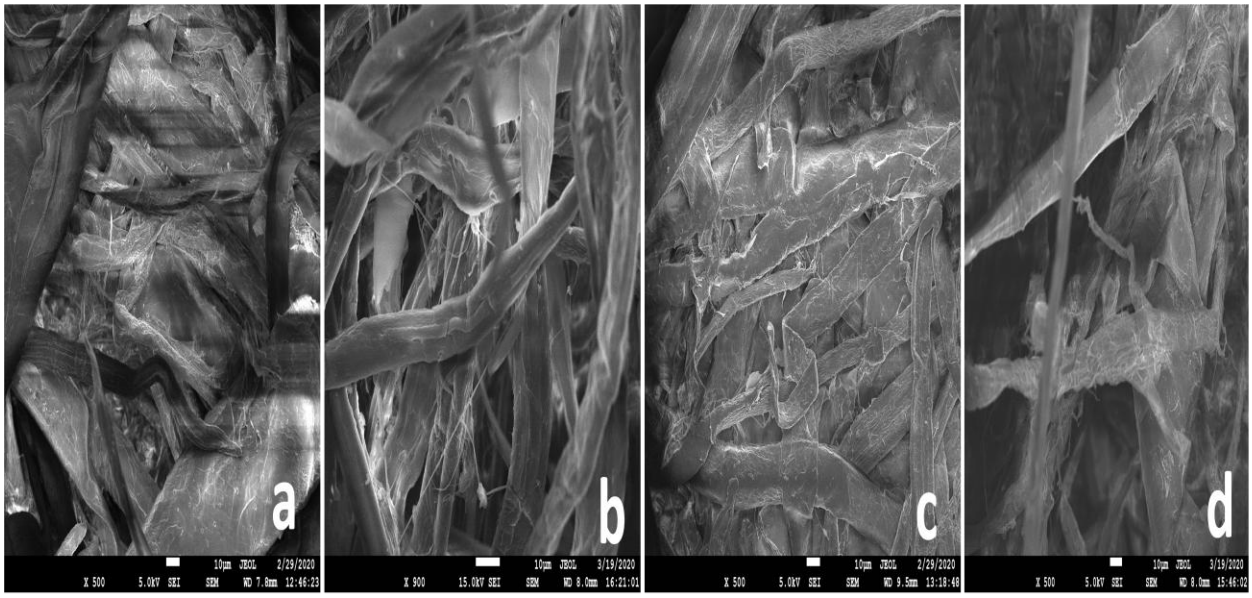


Fig. 6.36.0: Scanning electron microscope of (a) CMCase treated *F. oxysporum* VSTPDKF2 (b) CMCase treated *M. circinelloides* WSSDBS2F1, (c) Chemically treated and (d) control untreated pulps at x500 magnification

This development leads to an increase in surface area of fibre on the removal of water and hence may increase inter-fibre bonding resulting in an increased burst and tensile strength. During xylanase treatment, swelling, peeling and loosening of pulp fibre was observed by Kumar et al., (2018) when *E. coli* KD5 isolate produced cellulase-xylanase co production. Saini *et al.*, (2020) observed cracks and perforation when a pulp containing ozone and cellulase enzyme was viewed under scanning electron microscope. It was believed that the role of CMCase in cellulose degradation is to breakdown the cellulose fibres at different amorphous sites thereby act in the loosening of fibres that lead to the releasing of particles from waste paper during flotation with aid of a surfactant. An alkaline CMCase produced from *Fusarium sp* was also found to be very effective in the releasing of fibre from the surface of hairy toner which later improves the flotation efficiency by increasing the hydrophobicity of ink particles. Similar observations were reported on the fibrillation of pulp after enzyme treatment through a scanning electron microscope (Tsatsis *et al.,* 2017; Pathak *et al.,* 2014; Virk *et al.,* 2013).

An attenuated total reflection-Fourier transform infrared spectroscopy (FTIR) was carried out on enzyme deink pulp, chemically deink and control samples to examine the surface functional groups of the pulp. The FTIR spectra of chemical, control and enzyme deink pulp along with their control were recorded over a range of 4000-400cm^{-1} using 25 scans per sample at a resolution of 4cm^{-1}. This attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was used to observe the changes on the structural properties of enzyme deink, chemically deink and control pulp.

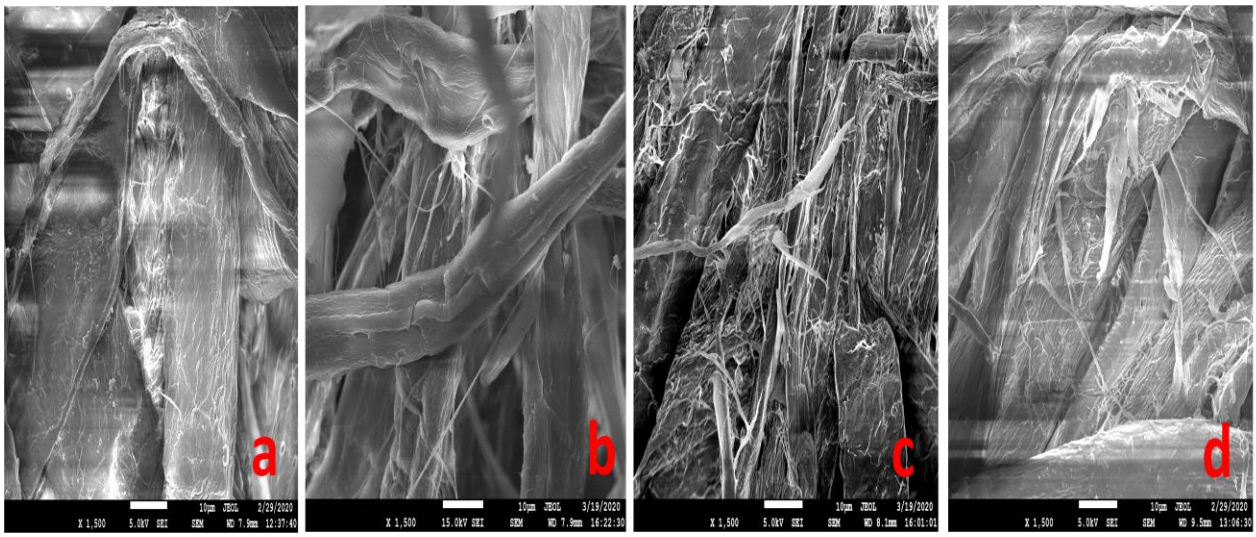


Fig. 6.37.0: Scanning electron microscope of (a) CMCase treated *F. oxysporum* VSTPDKF2 (b) CMCase treated *M. circinelloides* WSSDBS2F1, (c) Chemically treated and (d) control untreated pulps at x1500 magnification

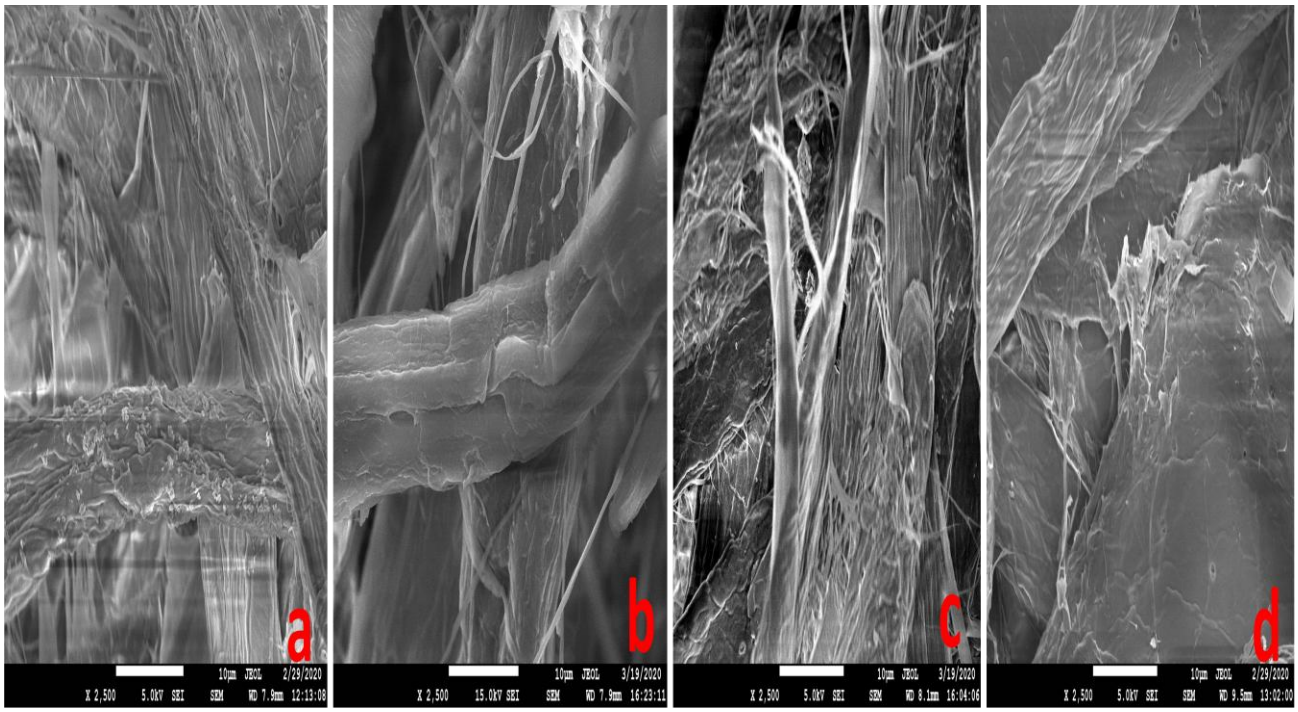


Fig. 6.38.0: Scanning electron microscope of (a) CMCase treated *F. oxysporum* VSTPDKF2 (b) CMCase treated *M. circinelloides* WSSDBS2F1, (c) Chemically treated and (d) control untreated pulps at x2500 magnification.

Enzyme treated pulp showed some similarities in the structural absorptions with chemical and control deink pulp but with the very clear difference in percentage intensity **(Fig. 6.39 to 6.44)**. There was a clear peak of the hydroxyl group (-OH) of cellulose between 3333cm^{-1} to 3335cm^{-1} for all samples but with different intensity as 93.67%, 88.64% for two enzyme deinking pulps (VSTPDKF2, WSSDBS2F1) as compared to 68.28% and 69.67% for chemically and control treated pulp respectively. This increase in intensity after enzymatic treatment is an indication of an increase in cellulosic content of the pulp. The decrease in intensity for all enzyme deinked pulp at a band between 3000cm^{-1} and 2917cm^{-1} which was assigned to carbonyl stretching of the saturated ketone is an indication of degraded aliphatic side chains. These peaks were not observed in control and chemically deink pulp and thus confirmed the effectiveness of enzymatic and physical deinking method as confirmed to chemical treatment. Virk *et al*., (2013) reported a decrease in relative intensity of bands at 2921cm^{-1}-2917cm^{-1} and 617 cm^{-1} assigned to CH asymmetrical stretching vibration in CH_3, CH_2, CH, in P-X-L-C treated pulp when a combine enzymatic deinking xylanase and laccase enzyme was carried out on recycled old newsprint. The peak between 1643 cm^{-1}-1316cm^{-1} implies the release of free carbonyl groups from the aromatic ring in enzyme treated pulp was less intense. Degradation of guaiacyl groups was also observed by the decrease in intensity on the enzyme deinked pulp between the bands of 1643cm^{-1} to 1316cm^{-1}. The FT-IR result also revealed another decrease in the band intensity which correspond to C=O=C bond at 557cm^{-1} from the two enzyme treated pulp as compared to chemical and control treated pulp is an indication of the presence of cellulose degradation. Saini *et al.,* (2020) revealed the significant changes in the chemical as well as the surface of the prepared hand sheet using SEM, TEM, FTIR and XRD analysis. They reported a similar decrease of intensity at 1270cm^{-1} on cellulase treated NP and ozone treated PP which confirmed the presence of degraded cellulose. Several papers have reported a similar result of less intensity when an enzyme treated pulp is subjected to ATR-FTIR spectroscopy as compared to chemical or control treated pulp (Desai and Iyer, 2016).

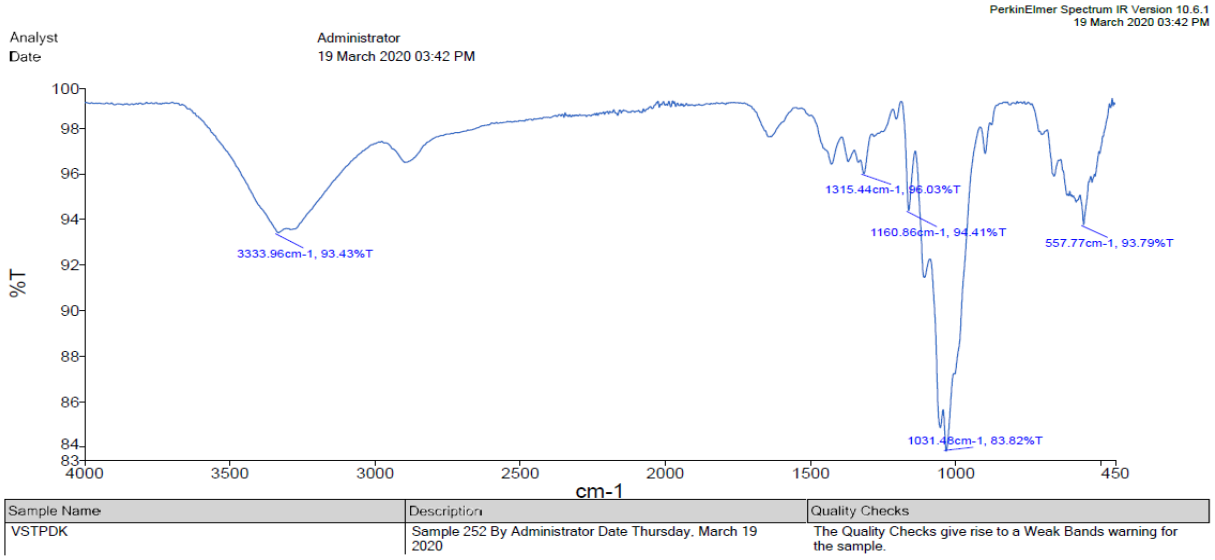


Fig. 6.39.0: The changes in the structure of pulp analyzed by FTIR spectroscopy from VSTPDKF2 enzyme treated sample

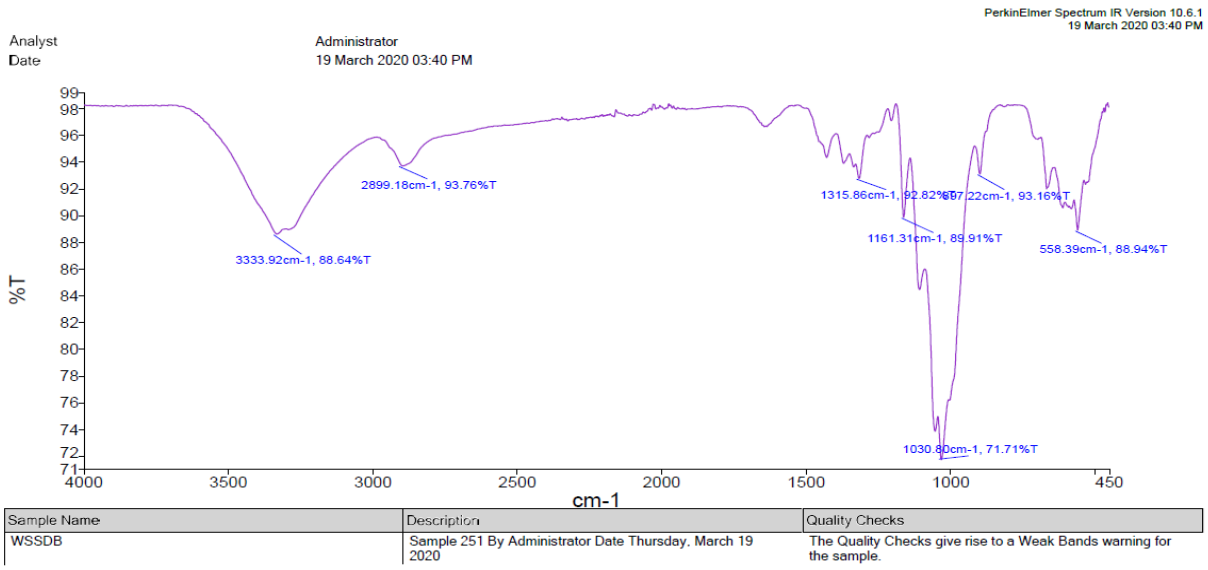


Fig. 6.40.0: The changes in the structure of pulp analyzed by FTIR spectroscopy from WSSDBS2F1 enzyme treated sample

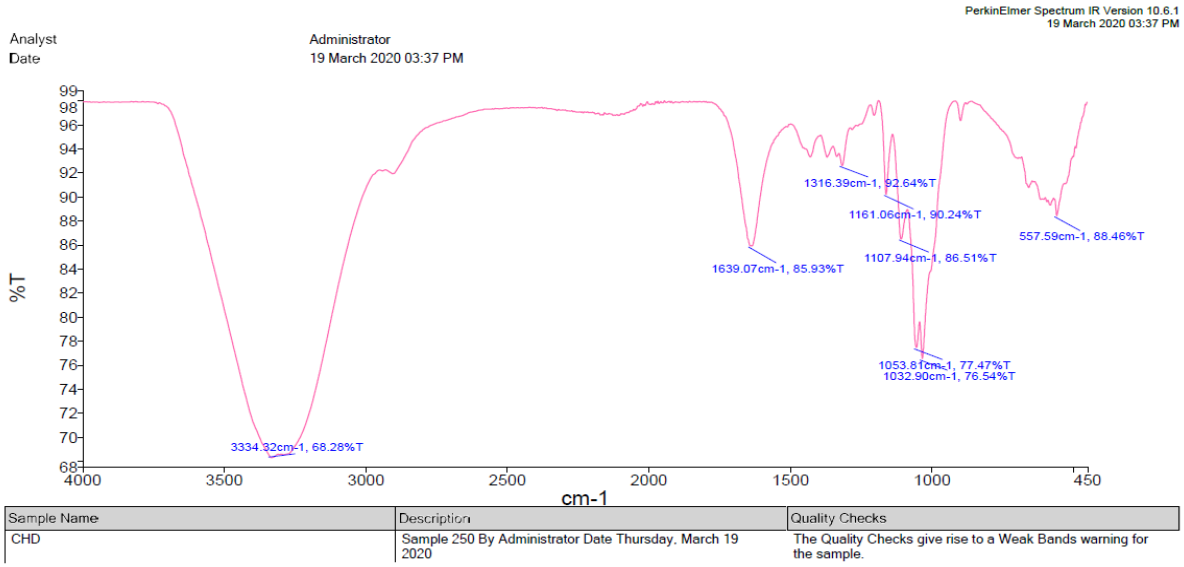


Fig. 6.41.0: The changes in the structure of pulp analyzed by FTIR spectroscopy from chemically deink treated sample

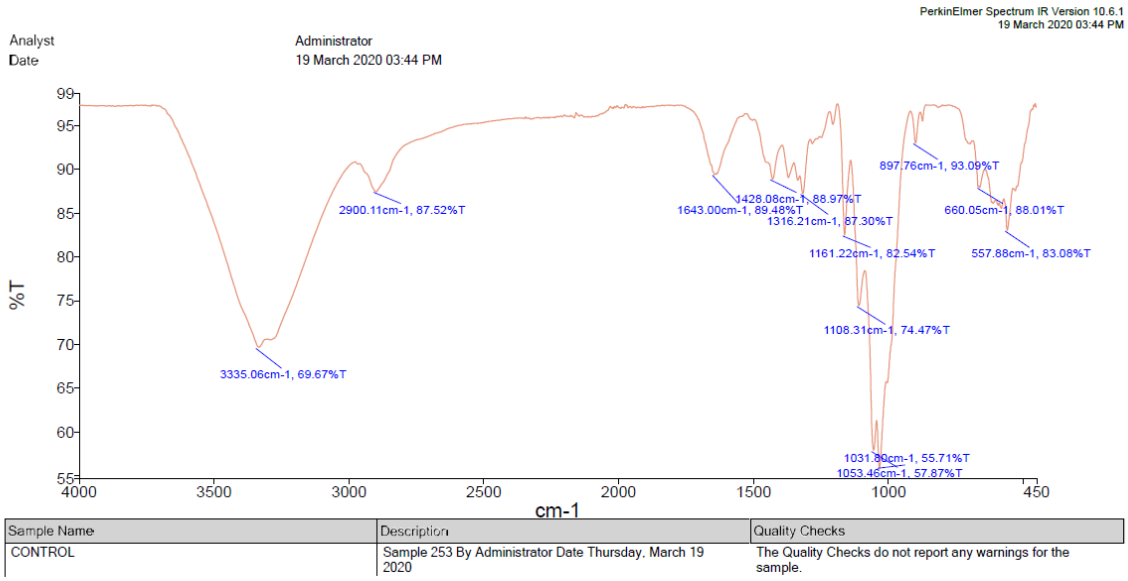
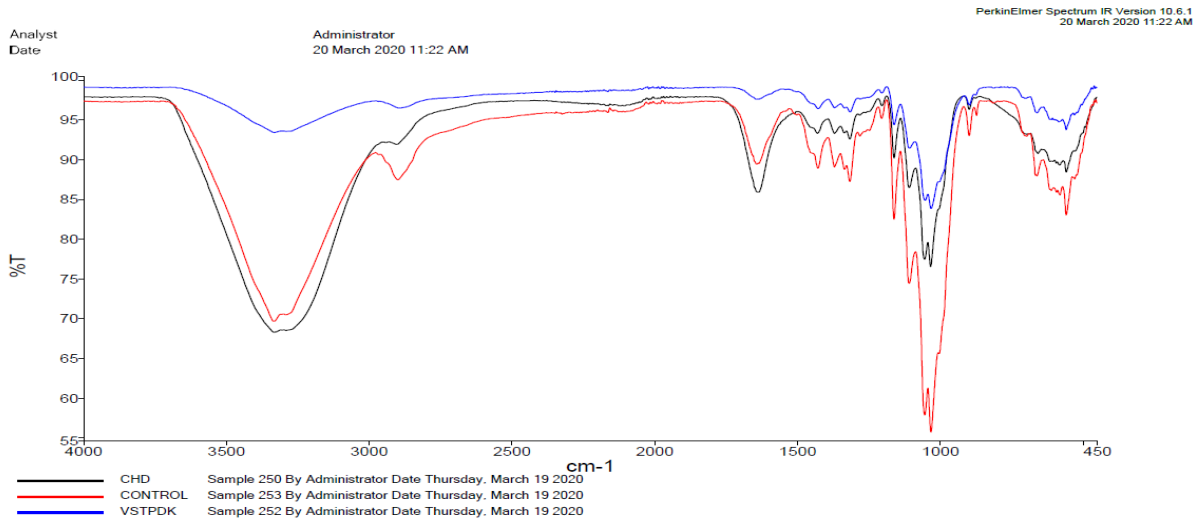
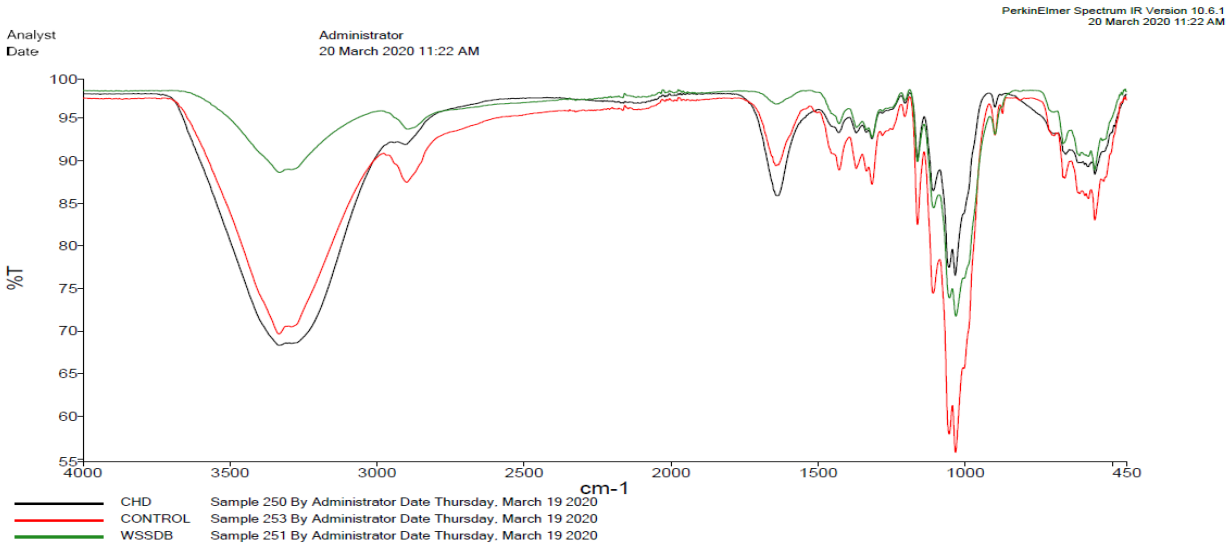


Fig. 6.42.0: The changes in the structure of pulp analyzed by FTIR spectroscopy from control sample



Key: **CHD means chemical deinking**

Fig. 6.43.0: The changes in the structure of the combine enzyme, chemical and control pulp samples analyzed by FTIR spectroscopy from *F. oxysporum* VSTPDKF2 treated



Key: **CHD means chemical deinking**

Fig. 6.44.0: The changes in the structure of the combine enzyme, chemical and control pulp samples analyzed by FTIR spectroscopy from *M. circinelloides* WSSDBS2F1 treated

CHAPTER SEVEN

Summary and Conclusion

7.1.0 Summary

Cellulase is one of the most important lignocellulolytic enzymes for bioconversion of cellulose to monosaccharides. Cellulase is a family of three (3) groups of enzymes called endo-(1,4)-β-D-glucanase(EC3.2.1.4), exo-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). Many microorganisms such as bacteria and fungi produce these cellulase enzymes when they grow on cellulosic materials. An excellent industrial enzyme is expected to possess high stability and activity in a wide range of fermentation conditions. These types of enzymes are mainly found in extreme environments. Over the past decades, attentions were focused on the exploration of alkaline enzymes for different biotechnological applications in industries ranging from paper, textile, food, detergent and biofuel. Despite these enormous applications, the industrial uses of these enzymes are affected by lack of microbial strains capable of generating a high amount of alkaline cellulase. Waste paper recycling is another way that can preserve green plants and environment thereby preventing environmental pollution, water and energy. The present conventional deinking method uses a large quantity of chemicals, chelating agents and surfactants capable of damaging the environment. The discovery on the use of enzyme for the deinking process was found to solve most of the problems associated with chemical deinking methods since it is environmentally friendly. Looking at the fact that India is one of the largest paper producing countries with large number of waste papers, several efforts were made to develop a method of deinking used papers through enzymatic activity. However, since the soil of Kapurthala district in Punjab was found to be in alkaline condition, the research was focused on isolating, screening and identifying prospective alkaliphilic fungi capable of producing alkaline cellulase enzymes for deinking properties.

7.1.1 Isolation and screening of alkaliphilic fungi

In this research, cellulose degrading alkaliphilic fungi were isolated from alkaline soil and some industrial effluents. Based on the research conducted, twenty-seven (27) alkaline fungi were isolated within the pH range of 7 to 11 using Mandel and Reese media with CMC and cellulose powder as a carbon source. From the twenty-seven (27) isolated fungi, seven fungi

showed a maximum clear hollow zone of hydrolysis in Mandel and Reese media containing CMC and cellulose powder as a carbon source using Congo red screening method. These seven selected fungi were subjected to extracellular cellulase enzyme activity on the same screening media supplemented with the same carbon sources for cellulase enzyme production. This crude alkaline enzyme was subjected to endoglucanase and exoglucanase enzyme assay using Dinitrosalicyclic acid (DNSA) method. Production of endoglucanase and exoglucanase activity from the seven alkaline fungal isolates shows that fungal isolate VSTPDKF2 has the highest mean enzyme activity followed by isolate WSSDBS2F1. The enzyme activity was found increase with an increase in incubation days from 4^{th} to 8^{th} day. However, a sudden decrease in enzyme activity was observed after 8^{th} day incubation up to 12^{th} day incubation. The two alkaline fungal isolates with the highest endoglucanase and exoglucanase activity were subjected to production and optimization using one factor at a time (OFAT) approach as well Response Surface Methodology (RSM) in order to optimize and identify the best nutritional as well as physicochemical parameters necessary for more enzyme production.

7.1.2 Identification of fungal cultures

The colony appearance on Mandel and Reese medium as well as Potato Dextrose Agar (PDA) and microscopic examination of the four fungal isolates with the highest activity were selected and identified as genera of *Fusarium* (VSTPDKF2), *Aspergillus* (VSTPDKF1), *Penicillium* (VRTPDKF1) and *Mucor* (WSSDBS2F1). Based on their molecular 18S rRNA gene identification, Integrated Transcribed Spacer (ITS1 5′-GTAGTCATATGCTTGTCTC-3′) and (ITS4 5′-CTTCCGTCAATTCCTTTAAG-3′) primers were amplified by 18S rRNA gene region, sequenced and analyzed for similarity among NCBI database. A BLAST search revealed VSTPDKF2 with 97.77% similarity with *Fusarium oxysporum* (Genbank accession number MT102254.1) and a phylogenetic tree was constructed using MEGA X software which the isolate was later assigned as *Fusarium oxysporum* VSTPDKF1. The second alkaline fungus designated as VSTPDK1 revealed 100% similarity with isolate *Aspergillus oryzae* (Genbank accession number MH782055.1). The similar identification process was carried out on our fungal isolate VRTPDKF1 isolated from the soil of Randhipura village in Kapurthala district where the gene sequencing revealed the isolate has a 99.71% similarity with *Penicillium chrysogenum* (GenBank accession number KX580630.1). The last isolate WSSDBS2F1 which was isolated

from sugarcane bagasse of Wahid Sandhar Sugar Ltd Phagwara was found to have 96.84% similarity with *Mucor circinilloides* (GenBank accession number HQ845293.1).

7.1.3 Optimization of culture conditions using OFAT and RSM

Low enzyme yield and substrate cost are some of the problems associated with cellulase production which mainly affect its large scale production. However, these limitations can be overcome by the application of optimizing parameters controlling enzyme yield. This can be done by either optimizing the physical factors like pH, incubation time and temperature or nutrient composition of the media such as carbon and nitrogen source. One factor at a time approach (OFAT) is a conventional method for enzyme production which is time-consuming, laborious and overlooks interactions between different variables, leading to misinterpretation of the data obtained. In order to examine the effects of various factors that influence responses by changing them simultaneously, a statistical approach called Response Surface Methodology (RSM) through central composite design (CCD) was used to replace one factor at a time (OFAT) approach.

7.1.4.1 Effect of initial pH

Being the cardinal factor of our research, the effects of pH on fungal growth and cellulase enzyme activity was studied between the ranges of 7 to 11. At alkaline pH, CMCase optimum activity (2.96 IU/ml and 2.59 IU/ml) and FPase activity (2.78 FPU/ml and 2.78 FPU/ml) from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 respectively were reported at pH 8. However, enzyme activity and stability on both CMCase and FPase from the two alkaline fungi were also recorded at pH 9 and 10. This enzyme activity at the alkaline environment is an indication that *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 can produce alkaline cellulase.

7.1.4.2 Effect of different Temperature

One of the essential physical factor affecting the survival and growth of microorganisms is temperature. The temperature for fungal growth and cellulase production was measured between the ranges of 30 to 60ºC. The optimum temperature for both *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 on CMCase and FPase was found to at 30^{O}C with activity

(3.50 IU/ml and 3.70 FPU/ml) and (3.32 IU/ml and 3.52 FPU/ml) respectively. Despite having an optimum activity of 30^{O}C, high temperature values also yield a good enzyme activity.

7.1.4.3 Effect of Incubation Time

Cellulase enzyme activity was determined based on their incubation period by inoculating the flasks on different incubation days (4^{th} - 12^{th}). It was observed that an initial increase in fermentation day lead to an increase in enzyme production. Optimum CMCase (3.52 IU/ml) and FPase activity (3.88 FPU/ml) from *F. oxysporum* VSTPDKF2 and *Mucor circinelloides* WSSDBS2F1 activity for CMCase (2.78 IU/ml) and FPase (3.88 FPU/ml) was observed on 8^{th} day incubation which subsequently declined with increase in the incubation period.

7.1.4.4 Effect of Different Carbon Sources

Alkali pre-treated lignocellulosic Rice straw (RS), wheat straw (WS) and sugarcane bagasse (SB) were selected and substitute carboxymethylcellulose (CMC) and cellulose powder as sole sources of carbon from the fermentation medium. It was found that CMCase (2.78 IU/ml) and FPase (3.70 FPU/ml) activity on rice straw from *F. oxysporum* VSTPDKF2 as well as CMCase (3.70 IU/ml) and FPase (3.89 FPU/ml) activity on sugarcane bagasse from *M. circinelloides* WSSDBS2F1 were optimum activity recorded on 8^{th} day incubation at 30^{O}C and pH 8.

7.1.4.5 Effect of Nitrogen Source

One of the most important nutritional factor influencing microbial growth and cellulase activity is the nitrogen source. Four different inorganic nitrogen sources which viz ammonium sulphate (NH_4SO_4), ammonium carbonate (NH_4HCO_3), ammonium chloride (NH_4Cl) and Sodium nitrate ($NaNO_3$) were used as a source of nitrogen. Maximum CMCase activity (3.50 IU/ml and 3.52 IU/ml) and FPase (3.70 FPU/ml and 3.50 FPU/ml) from *F. oxysporum* VSTPDKF2 and *M. circinelloide*s WSSDBS2F1 respectively were reported from ammonium sulphate (NH_4SO_4).

7.1.4.6 Effect of Ammonium Sulphate Concentration

Effect of various ammonium sulphate concentrations on alkaline cellulase activity was also investigated. The result obtained indicated that when 1% to 5% concentration was used, 3.0% was found as optimum concentration supporting cellulase activity form both alkaline *F. oxysporum* VSTPDKF2 CMCase and FPase (3.70 IU/ml and 3.89 FPU/ml) and *M. circinelloides* WSSDBS2F1 CMCase and FPase (3.52 IU/ml and 3.33 FPU/ml) respectively. Increase or decrease of ammonium sulphate from the optimum concentration was found to lower the rate of enzyme activity.

7.1.5 Central Composite Design (CCD) of Experiment

Three important parameters identified following enzyme production from one factor at a time (OFAT) were further optimized using CCD in Response System Methodology (RSM). A total of twenty (20) experiments each from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 alkaliphilic fungi were randomly designed by the software and later conducted in our laboratory as predicted. The independent variables were pH (A), temperature (B) and concentration of ammonium sulphate (C). Estimated endoglucanase and exoglucanase as a function of independent variables were obtained after ANOVA has provided a regression equation. Models precisions were normally determined by a coefficient (R^2). Its values always range between 0 to 1 and the order of magnitude suggests goodness of the model. The R^2 values of CMCase and FPase from *F. oxysporum* VSTPDKF2 were found to be 0.9933 and 0.9894 respectively while R^2 values of CMCase and FPase enzymes produced from *M. circinelloides* WSSDBS2F1 are 0.9852 and 0.9899 respectively. These R^2 values are close to 1 which signifies that the 99.33% and 98.94 behavior of the model from *F. oxysporum* VSTPDKF2 as well as 98.52% and 98.99% behavior of the from *M. circinelloides* WSSDBS2F1 can be interpreted for alkaline cellulase enzyme production while only 0.67% and 1.06%, as well as 1.48% and 1.01% full variance, cannot be explained by the models. The Adjusted R^2 from this model was 0.9873 and 0.9798 as well as 0.9721 and 0.9808 for the two fungal isolates respectively. This is an indication of a good relationship between actual and predicted values. From the result obtained, predicted R^2 values for CMCase (0.9710 and 0.9388) and FPase (0.9300 and 0.9279) agreed with Adjusted R^2 values of 0.9873 and 0.9798 respectively. Hence, the model provides clarity on the relationship between response and independence variables. Adequate precisions of the model

measured the signal to noise ratio for CMCase and FPase from *F. oxysporum* VSTPDKF2 (48.790 and 39.373) and *M. circinelloides* WSSDBS2F1 (31.58 and 36.622) respectively indicated an adequate signal while the result showed that the model is significant.

Significant of the models are generally measured on the basis of P-value of F-value (prob > F). The result indicated that high models *F*-value for alkaline CMCase and FPase produced from *F. oxysporum* STPDK are 165.31 and 103.28 with their respective small prob >F values (P-value) of <0.0001 signify that the models were significant. Meanwhile, CMCase and FPase produced from alkaline *M. circinelloides* WSSDBS2F1 recorded an *F*-value of 74.56 and 109.01 respectively with their corresponding prob >F values (P-value) of <0.0001 indicated that that the model is significant. The prob>F<0.05 values showed that the models were significant. This means that A, B, C, A^2, B^2, C^2, AB, AC as well as BC are the significant model terms. The lack of fit *F*-value of the models for both CMCase and FPase from *F. oxysporum* VSTPDKF2 are 1.83 and 4.29 while lack of fit P-values were 0.2613 and 0.0679 respectively while the lack of fit F-value of the models for CMCase and FPase from alkaline *M. circinelloides* WSSDBS2F1 are 2.80 and 4.93 with their corresponding lack of fit P-value as 0.1414 and 0.0524 respectively indicated that lack of fits was not significant and that models are very accurate without any noise. The 3D response surface, as well as their contour plots in central composite design (CCD), shows an interaction between the two different factors while keeping other factors constant. This visualization helps to understand the interaction between two factors and pinpoint the optimum level of each parameter for maximal response. The interaction between ammonium sulphate concentration and enzyme production showed an important effect. Increases in ammonium sulphate concentration will increases enzyme production from 1% to 3% and where subsequently increase showed a decrease in enzyme production. The optimum enzyme production was indicated at 3% ammonium sulphate and pH 8.5. An excellent correlation was also observed between pH and temperature as well as temperature and ammonium sulphate concentration.

7.1.6 Validation of Statistical Experiments

The accurate prediction and model's fitness evaluation of each variable investigated requires validation of the model from both a graphical and numeric approach. To examine the significance of the facts, simple statistical and mathematical tests such as F test, P-value and ANOVA were used in validating the experimental design. Based on the RSM-CCD results, the

optimum conditions for enzyme production were 8.5% initial pH, 45^{O}C temperature and 3% ammonium sulphate concentration, while incubation time was kept constant (8 days). From alkaline, *F. oxysporum* VSTPDKF2, RSM experiments reported an actual enzyme production of 3.91 IU/ml and 4.26 FPU/ml for CMCase and FPase respectively. While from *M. circinelloides* WSSDBS2F1, both CMCase and FPase reported an actual enzyme production of 4.26 IU/ml. These experiments were conducted based on the different predicted experiments from three factors identified as pH, temperature and NH_4SO_4 concentration in order to validate the results previously conducted with CCD. Comparison between OFAT and RSM from *F. oxysporum* VSTPDKF2 revealed an increased enzyme activity of 0.39 IU/ml (CMCase) and 0.19 FPU/ml (FPase) using RSM as compared to that of OFAT. Similarly, *M. circinelloides* WSSDBS2F1 RSM result revealed an increased enzyme activity of 0.74U/ml and 0.56U/ml for CMCase and FPase respectively as compared to OFAT. This indicated that RSM can be used to increase cellulase enzyme production as compared to OFAT and other conventional methods of enzyme productions.

7.1.7 Evaluation of Endoglucanase Enzyme Deinking Potentials

The present conventional deinking method uses chemicals such as sodium hydroxide, sodium silicate, sodium carbonate and hydrogen peroxide that are usually hazardous. Alternatively, enzymatic deinking can be used to replace the conventional method with little environmental hazard and energy saving. The application of enzymes has been suggested as an environmentally friendly alternative way of replacing conventional deinking in the recycling waste paper. In this research, an A4 paper was printed with the word ‘deinking’ for approximately 640 words using xerox printer. After enzymatic, chemical and control pulping process as well as flotation in our laboratory, the resultant pulp was prepared according to TAPPI standard and sent to the Central Pulp & Paper Research Institute (CPPRI) Saharanpur, 247001 (U.P.) India for the testing of pulp brightness, tensile strength, bursting strength as well as tearing strength.

The result shows the optical and mechanical properties of the prepared hand sheet which includes percentage brightness, tensile strength, bursting strength and tearing strength after enzymatic treatment with crude alkaline endoglucanase isolated from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 as well as chemical deinking and control. The percentage

brightness of 81.99% and 81.63 were recorded for *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 respectively as compared to 81.51% and 79.63% for chemical treatment and control respectively. The percentage brightness result following *F. oxysporum* VSTPDKF2 enzymatic treatment indicated an increase ISO brightness of 2.36% and 0.48% on control and chemical respectively from the prepared hand sheet. Moreover, 1.00% and 0.12% improvement in ISO brightness were recorded following enzymatic treatment of CMCase from *M. circinelloides* WSSDBS2F1. The mechanical properties of the hand sheet for alkaline CMCase isolated from *F. oxysporum* VSTPDKF2 includes 1080N/m tensile strength, 59KPa burst strength and 288mN tear index while that of *M. circinelloides* WSSDBS2F1 are 1250N/m tensile strength, 67KPa burst strength and 352mN tear index. The mechanical properties of control and chemical deinking experiment include tensile strength (1070 and 953N/m), bursting strength (56 and 58KPa) as well as tear index 285 and 279mN respectively. Enzymatic deinking using CMCase produced from *F. oxysporu*m VSTPDKF2 and *M. circinelloides* WSSDBS2F1 improved the tensile strength of the printed waste paper by 10N/m and 127N/m as well as 180N/m and 253N/m respectively when compared to control and chemical deinking process. In terms of bursting strength, enzymatic deinking of the enzyme produced by these isolated fungi increase the bursting strength of the deink hand sheet by 3KPa and 1KPa from *F. oxysporu*m VSTPDKF2 and also 11KPa and 8KPa from *M. circinelloides* WSSDBS2F1 when compared to control and chemical deinking respectively. The third mechanical property of the prepared hand sheet tested was tearing strength **w**hich shows an improvement of 3mN and 9mN as well as 11mN and 8mN following enzymatic deinking alkaline CMCase isolated from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 respectively as compared to control and chemical deinking process respectively.

In order to confirm the influence of these enzymes for potential deinking ability, the prepared pulp was subjected to Scanning Electron Microscope (SEM) and Fourier Transformed Infrared Spectroscopy (FTIRS). It is observed that enzymatic action usually resulted in internal fibrillation of fibre as well as some degree of surface fibrillation in the fibre. Increased strength can be signed to internal as well as surface fibrillation of the fibre by enzymatic action through a Scanning Electron Microscope (SEM) which can be clearly seen as compared to chemically deink and control untreated pulp. SEM pulp analysis revealed some changes such as crack development, pores as well as peeling off fibre. Fibre swelling, loosing and peeling off of fibre

are clearly visible on enzymatic deink pulp when compared with control and chemically deink pulp. This development leads to an increase in surface area of fibre on the removal of water and hence may increase inter-fibre bonding resulting in an increased burst and tensile strength. An attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was carried out on enzyme deink pulp, chemically deink and control samples in order to examine the surface functional groups of the pulp. The FTIR spectra of chemical, control and enzyme deink pulp along with their control were recorded over a range of 4000-400cm^{-1} using 25 scans per sample at a resolution of 4cm^{-1}. This ATR-FTIR was used to observe the changes in the structural properties of enzyme deink, chemically deink and control pulp. Enzyme treated pulp showed some similarities in the structural absorptions with chemical and control deink pulp but with a very clear difference in percentage transmittance. There was the clear peak of the hydroxyl group (-OH) of cellulose between 3333cm^{-1} to 3335cm^{-1} for all samples but with different transmittance as 93.67%, 88.64% for two enzymes deinking pulp as compared to 68.28% and 69.67% for chemically and control-treated pulp respectively. This increase in percentage transmittance after enzymatic treatment is an indication of an increase in cellulosic content of the pulp. The decrease of transmittance for all enzyme deinked pulp at a band between 3000cm^{-1} and 2917cm^{-1} which was assigned to carbonyl stretching of the saturated ketone is an indication of degraded aliphatic side chains. These peaks were not observed in control and chemically deink pulp and thus confirmed the effectiveness of enzymatic and physical deinking method as confirmed to chemical treatment. The peak between 1643 cm^{-1}-1316cm^{-1} implies the release of free carbonyl groups from the aromatic ring in enzyme treated pulp was less intense. Degradation of guaiacyl groups was also observed by the decrease in intensity on the enzyme deinked pulp between the bands of 1643cm^{-1} to 1316cm^{-1}. The FT-IR result also revealed another decrease in the band intensity which correspond to C=O=C bond at 557cm^{-1} from the two enzyme treated pulp as compared to chemical and control treated pulp is an indication of the presence of cellulose degradation.

7.2.0 Conclusion

During the last decades, alkaliphilic microorganisms have attracted attentions for isolating, screening, identifying and exploring their potentials for biotechnological applications and environmental pollution mitigation. Many of such alkaliphilic microorganisms have been isolated from a wider range of alkaline environments ranging from alkaline soda lakes and soil.

The aim of this research was to search for prospecting alkaline cellulase producing fungi from the soil and industrial effluents from Kapurthala as well as Amritsar districts, Punjab. Mandel and Reese screening media was used to isolate twenty-seven potential alkaline cellulase producing fungi from eight soil samples, industrial effluent from Khanna Paper Mills Amritsar and Wahid Sandhar sugar Ltd Phagwara. Of the twenty-seven fungi isolated, seven fungal cultures (VSTPDKF2, VSTPDKF1, VRTPDKF1, VNTPDKF2, KPMAS2F1, KPMAS4F1 and WSSDBS2F1) were found to positive cellulase enzyme activity in Congo red screening. These seven alkaline fungal cultures were subjected to cellulase enzyme production in submerged fermentation technique for 12 days incubation at 30^{O}C temperature, 8 pH using Mandel and Reese media with carboxymethylcellulose as carbon source. Four of these cultures with highest enzyme activity (VSTPDKF2, VSTPDKF1, VRTPDKF1 and WSSDBS2F1) were selected and identified morphologically, microscopically and using 18S rRNA gene sequencing as *Fusarium oxysporum* VSTPDKF2, *Aspergillus oryzae* VSTPDKF1, *Penicillium chrysogenum* VRTPDKF1 and *Mucor circinelloides* WSSDBS2F1. The two alkaline fungal isolates *Fusarium oxysporum* VSTPDKF2 and *Mucor circinelloides* WSSDBS2F1 that produced highest cellulase enzyme were further selected for endoglucanase and exoglucanase activity using one factor at a time approach (OFAT) at different physicochemical and nutritional parameters which includes pH, temperature, incubation time, different carbon source and different nitrogen source. In an effort to improve alkaline cellulase enzyme production, optimization of three important parameters which includes pH, temperature and ammonium sulphate concentration were conducted with Response Surface Methodology (RSM) software through Central Composite Design (CCD) of the experiment. Twenty (20) different predicted experiments designed by the software were carried out in the laboratory for the production of the actual enzyme. A one way ANOVA was used for the analysis of the experimental results of both CMCase and FPase of the two alkaliphilic fungi. Validation of the experimental result from RSM confirmed an increase of CMCase and FPase enzyme productions from both fungal isolates as compared to OFAT. This is an indication of the application of response surface methodology (RSM) on the improvement of cellulase enzyme production as compared to conventional methods.

Endoglucanase enzyme produced from *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 were used for the removal of ink from waste paper. An A4 paper was printed with word ‘deinking’ for approximately 640 words using xerox printer and used for deinking analysis.

After enzymatic, chemical and control deinking process as well as flotation, the resultant pulp was prepared according to TAPPI standard and sent to Central Pulp & Paper Research Institute (CPPRI) Saharanpur, 247001 (U.P.) India for the testing of pulp brightness, tensile strength, bursting strength as well as tearing strength. Enzymatic deinking with these two alkaline fungi have shown an improvement of both optical and mechanical strength as compared to chemical and control treated pulp. These improvements in brightness and mechanical properties of the enzyme treated pulp were confirmed by fibrillation and swelling of the fibre as observed using a Scanning Electron Microscope (SEM). An attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was also used to confirm the action these alkaline cellulase enzymes on treated pulp by observing changes in the structural properties of the enzyme treated pulp as compared to chemical and control pulp. Based on the research conducted, it was concluded that a novel alkaline cellulase producing *F. oxysporum* VSTPDKF2 and *M. circinelloides* WSSDBS2F1 were isolated, screened and identified. CMCase produced from these fungi was evaluated and confirmed their potential application in deinking of waste paper as such, can be used as a potential replacement of conventional deinking method in pulp and paper industry.

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APPENDIX-I

ABBREVIATIONS

S/No.	Abbreviation	Full Meaning
1	%	Percentage
2	≥	Greater or equal
3	>	Greater than
4	<	Less than
5	-	Minus
6	+	Plus
7	()	Open and close bracket
8	E	Exponential
9	β	Beta
10	µg	Microgram
11	±	Plus or Minus
12	^{O}C	Degree centigrade
13	IU/ml	International Unit per Millilitre
14	FPU/ml	Filter Paper Unit per millilitre
15	API	Analytical Profile Index
16	CMC	Carboxymethyl Cellulose
17	OFAT	One Factor At a Time
18	RSM	Response Surface Methodology
19	cm	Centimetre
20	FTIR	Fourier transform infrared spectroscopy
21	ART	attenuated total reflection
22	SEM	scanning electron microscope
23	KPa	Kilopascal
24	N/m	Force per unit length
25	mN	Millinewtons

26	U.P	Utra Pradsh
27	COD	Chemical Oxygen Demand
28	TAPPI	Technical Association of the Pulp and Paper Industry
29	CCD	Central Composite Design
30	R^2	Coefficient
31	RS	Rice Straw
32	WS	Wheat Straw
33	SB	Sugarcane bagasse
34	NCBI	National Center for Biotechnology Information
35	ITS	Integrated Transcribed Spencer
36	ANOVA	Analysis Of Variance
37	DNA	Deoxyribonucleic Acid
38	rRNA	Ribosomal Ribonucleic Acid
39	PCR	Polymerase Chain Reaction
40	mm	Millimetre
41	CP	Cellulose Powder
42	CIF	Central Instrumentation facility
43	g/L	Gram per litre
44	w/v	Weight by volume
45	VDTPDK1	Village Domeli Tehsil Phagwara District Kapurthala
46	VDTPDK2	Village Dhoda Tehsil Phagwara District Kapurthala
47	VUTPDK	Village Ucha Tehsil Phagwara District Kapurthala
48	VRTPDK	Village Randhipura Tehsil Phagwara District Kapurthala
49	VFTPDK1	Village Fattudhinga Tehsil Phagwara District Kapurthala
50	VNTPDK	Village Natpura Tehsil Phagwara District Kapurthala
51	VFTPDK2	Village Fatehpura Tehsil Phagwara District Kapurthala
52	VSTPDK	Village Samana Tehsil Phagwara District Kapurthala
53	KPMA	Khanna Paper Mills Amritsar
54	WSSDB	Wahid Sandhar Sugar Decomposing Bagasse
55	S	Sample

56	F	Fungus
57	lbs	Libra Balance Scale
58	N	Normality
59	M	Morality
60	AD	After Death
61	sp	Specie
62	ONP	Old News Print
63	CPPRI	Central Pulp & Paper Research Institute
64	Ltd	Limited
65	rpm	Revolution Per Minute
66	Min	Minute
67	nm	Nanometer
68	mg	Milligram

APPENDIX II

LIST OF CHEMICALS USED IN THE RESEARCH

S/NO.	Chemicals
1	2, 4 Dinitrophenol
2	Agar-agar
3	Acetone
4	Acetic Acid
5	Ammonium Oxalate
6	Ammonium Hydrogen Phosphate
7	Ammonium Nitrate
8	Ammonium Sulphate
9	Carboxymethyl Cellulose
10	Calcium Chloride
11	Cellulose Powder
12	Congo Red
13	Deionized water
14	Dinitro Salicyclic Acid
15	Dipotassium Hydrogen Phosphate
16	Disodium Phosphate
17	Ethylene Diamine Tetra Acetic Acid
18	Ferrous Sulfate
19	Ferric Chloride
20	Hydrogen Disulfate
21	Hydrogen Peroxide
22	Hydrochloric Acid
23	Potassium Dihydrogen phosphate
24	Potassium Chloride
25	Sodium Acetate
26	Sodium Chloride

27	Sodium Carbonate
28	Sodium Potassium Tartarate
29	Sodium nitrate
30	Sodium Hydroxide
31	Sodium Metabisulfite
32	Sodium Citrate
33	Manganese Sulfate
34	Magnesium Sulfate
35	Proteose Peptone
36	Peptone
37	Potassium Hydroxide
38	Tetra Methyl P Phenylene Diamine Dihydrochloride
39	Tween80
40	Zinc Chloride

INSTRUMENTS

S/No.	**Instruments**
1	BOD Incubator MAC
2	Cooling Centrifuge
3	Compound Light Microscope
4	High precision water bath with digital temperature controller
5	Oven
6	Laminar flow cabinet
7	Remi Auto Mix blender
8	Microprocessor visible spectrophotometer LI-721 model
9	Microwave 30SC3
10	Microprocessor Digital pH meter
11	Refrigerator
12	Weighing balance LCD series
13	Wire-mess 220

NAMES OF MICROORGANISMS AND THEIR ABBREVIATIONS

S/No.	Abbreviation	Full Meaning
1	*A. flavipes*	*Aspergillus flavipes*
2	*A. carneus*	*Aspergillus carneus*
3	*A. flavus*	*Aspergillus flavus*
4	*A. tamari*	*Aspergillus tamari*
5	*A. oryzae*	*Aspergillus oryzae*
6	*A. parasiticus*	*Aspergillus parasiticus*
7	*A. niger*	*Aspergillus niger*
8	*F. solani*	*Fusarium solani*
9	*F. oxysporum*	*Fusarium oxysporum*
10	*F. chlamydosporum*	*Fusarium chlamydosporum*
11	*F. verticilloides*	*Fusarium*
12	*F. equiseti*	*Fusarium equiseti*
13	*F. proliferatum*	*Fusarium proliferatum*
14	*M. circinelloides*	*Mucor circinelloides*
15	*P. citrinum*	*Penicillium citrinum*
16	*P. chrysogenum*	*Penicillium chrysogenum*

APPENDIX III

SEQUENCE OF MICROORGANISMS IDENTIFIED IN THIS RESEARCH USING 18S rRNA SEQUENCING

Forward sequence of *Fusarium oxysporum*

>VSTPDK_NS1_B09.ab1

TACCCGCGAAACTGCGAATGGCTCATTATATAAGTTATCGTTTATTTGATAGTACCTT
ACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCGACTTC
GGAAGGGATGTATTTATTAGATTAAAAACCAATGCCCTTCGGGGCTCACTGGTGATT
CATGATAACTCCTCGAATCGCATGGCCTTGTGCCGGCGATGGTTCATTCAAATTTCTT
CCCTATCAACTTTCGATGTTTGGGTATTGGCCAAACATGGTTGCAACGGGTAACGGA
GGGTTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGA
AGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAAT
ACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTT
AACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTC
CAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAACCTTGGGCCT
GGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCCCTCTGTGG
AACCCCATGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTTTTACTGTGAAAAAAT
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GACGTGTGGTTCTATTTTGTTGGTGTCTACGACCGCCCTCATGATTATTAGGGACAGT
CAGTGGCATCAGTATTCACTTGTCAGAGGTGAAATTCTTGGATGTATTGAAAACTAA
CTACTGCGACGCCGTTAGCGAGGATGTTTTCATTATTAAGAACGACCGTACGGG

Forward sequence of *Penicillium chrysogenum*

>VST_PDK1_NS1_H11.ab1

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AAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTTGGTGATTCA
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CCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGG
GAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGA

AGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAAT
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AACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTC
CAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGTCT
GGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTCCTTCTGGGG
AACCTCATGGCCTTCACTGGCTGTGGGGGGAACCAGGACTTTTACTGTGAAAAAATT
AGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATTAGCATGGAATAATAGAATAGG
ACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGT
CGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAA
CTACTGCGAAAGCATTCGCCAAGGATGTTTTCTTTAATCAGGGAACGAAAGTTAGGG
GATCGAAGACGATCAGATACCGTCGTAGTCTTACCATAAACTATGCCGACTAGGGAT
CGGGCGGTGTTTCTATGATGACCCGCTCGGACCTTCCAGAAATCAAGT

Forward sequence of *Aspergillus oryzae*

>VRT_PDK_NS1_G11.ab1

GGAAACTGGCGAATGGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACT
ACATGGATACCTGTGGTAATTCTAGAGCTAATACATGCTAAAAACCCCGACTTCAGG
AAGGGGTGTATTTATTAGATAAAAAACCAACGCCCTTCGGGGCTCCTTGGTGAATCA
TAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGC
CCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGG
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TCGGACGGGATTCTATAATGACCCGTTCGGCACTTACGAGAAATCAAAGTTTTTGGG
TTCTGGGGGGAATATGGTCCCAGGCTGAACTTAAGGAATTACGAGAAAACGCGAAA
AGGGGGCGG

Forward sequence of *Mucor cerciniloides*

>WSSDB_NS1_E10.ab1

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ACTACTGTAGGATAGCCGTGGTACTTCAACAGCTACTAGCTGCAAATCAACACTTAC
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GTGGATTCTCCATAATTACGCGGATCGCATGGCCTTGTGCTAGCGACAGTCCACTCT
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GACCAAAACAACTACTGCGAAGCATTGATCCCGGACTTTTCATTGATCAAGGCCAGA
GTTAGGGATCGACACGATCTGATCCGTCCTATCCTACCACGACCTTGCCGACTAAAA
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APPENDIX IV

PREPARED REAGENTS

Preparation of Buffer Solution

(i) Citrate Buffer

Stock Solution

0.1 M solution of Citric Acid = 21.01 gm in 1 lt. of distilled water

0.1 M solution of Sodium Citrate = 29.4 gm in 1 lt. of distilled water

pH	0.1 M Citric Acid (in ml.)	0.1 M Sodium Citrate (in ml.)
3.0	16.4	3.6
3.4	14.6	5.4
3.8	12.7	7.3
4.2	10.8	9.2
4.6	8.9	11.1
5.0	7.0	13.0
5.4	5.1	14.9

Preparation of Di-Nitro Salicyclic Acid (DNS) Reagent

3, 5 di-nitro salicyclic acid reagent was prepared for determining the reducing sugar as per the method of Miller (1959).

- Distilled water - 1416 ml
- 3, 5 di-nitro salicyclic reagent - 10.6 gm

- Sodium hydroxide - 19.8 gm

Dissolve the above chemicals, and then add

- Sodium Potassium Tartarate (Rochelle salt) - 306 gm
- Phenol (melted at 50^0C) - 7.6 ml
- Sodium metabisulfite - 8.3 gm

This reagent should be stored at 4^0C in Stoppard bottle until used for enzymatic activity.

Original report of hand sheets optical and mechanical properties after enzymatic, chemical and control flotation from Central Pulp & Paper Research Institute (CPPRI), UP India.

केन्द्रीय लुग्दी एवं कागज अनुसंधान संस्थान
Central Pulp & Paper Research Institute
An autonomous organisation under the administrative control of Ministry of Commerce & Industry, Govt. Of India
(Registered under Societies Act)

CPPRI/ SPPM Div/2019-20/R-80 19- March- 2020

TEST REPORT

Sample received from: Mr Abdulhadi Yakubu
C/o Dr Ashish Vyas
Room no 28-202 cabin 2
Department of Microbiology School of Bioengineering and Biosciences, Delhi GT Road
Lovely Professional University
Phagwara (Punjab) - 144 411

Ref. No. & Date: Email dated 21.02.2020

Sample Detail: Deinked Pulp Samples (Control, Chemical, VSTPDK & WSSDB)

Testing Required: Testing of Deinked Pulp Samples for Brightness, Tensile strength, Bursting Strength and Tearing Strength

Test Result

S. No	Test Parameter	Unit	Value				Testing Reference Method
			Control	Chemical	VSTPDK	WSSDB	
1	Brightness	%	79.63	81.51	81.99	81.63	IS/ISO 2470-1:2009 (RA.2013)
2	Tensile Strength	N/m	1070	953	1080	1250	IS 1060 (Part 5/ Sec 6):2014
3	Bursting Strength	kPa	56	58	59	67	IS 1060 (Part 6/ Sec 2):2014
4	Tearing Strength	mN	285	279	288	352	IS 1060 (Part 6/ Sec 1):2014

(Rita Tandon)
Scientist-G & Head
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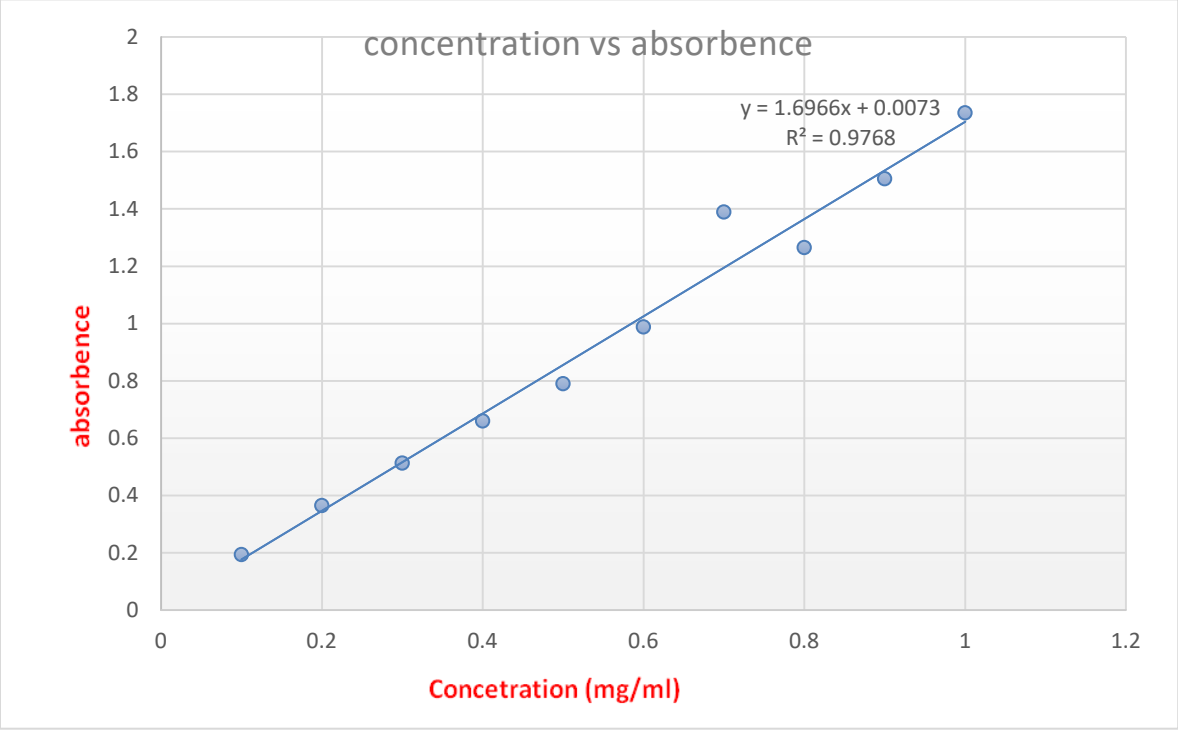
BASE OFFICE
A-55, 3rd Floor, Gujranwala Town, Part-1, Near Vinayak Hospital, Delhi-110 009
Phone - (011)-49027213, 9910909169

Glucose standard table used estimating the amount enzyme at 540nm

Tubes	Glucose concentration (mg/ml)	Absorbance
Black	0.0	0.000
1	0.1	0.194
2	0.2	0.365
3	0.3	0.513
4	0.4	0.660
5	**0.5**	**0.790**
6	0.6	0.988
7	0.7	1.389
8	0.8	1.265
9	0.9	1.505
10	1.0	1.735



Optical density of glucose standard curve

APPENDIX V

LIST OF PUBLICATION AND PRESENTATIONS

Publication

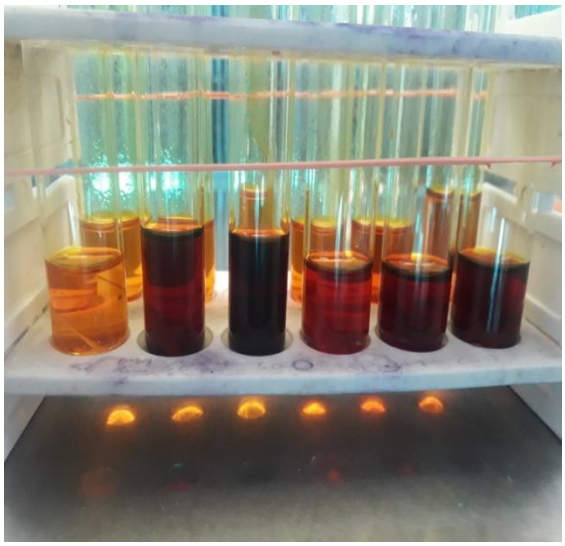
1. Book chapter published in Springer titled "**MICROBIAL ENZYMES AND THEIR APPLICATION IN PULP AND PAPER INDUSTRY**" A chapter 12 in Recent Advancement of White Biotechnology through Fungi. Volume 3: Perspective for sustainable environments, (2019): 297-317 DOI https://doi.org/10.1007/978-3-030-25506-0_12 link *Microbial enzymes and their Application in Pulp and Paper Industry*
2. Accepted paper for publication titled **"RESPONSE SURFACE METHODOLOGY APPROACH FOR OPTIMIZATION OF ENDOGLUCANASE FROM ALKALIPHILIC *FUSARIUM OXYSPORUM* VSTPDK AND ITS POTENTIAL APPLICATION IN PULP AND PAPER INDUSTRIES** to be published in *Research Journal of Biotechnology*. Indexed in Scopus and Web of Science.

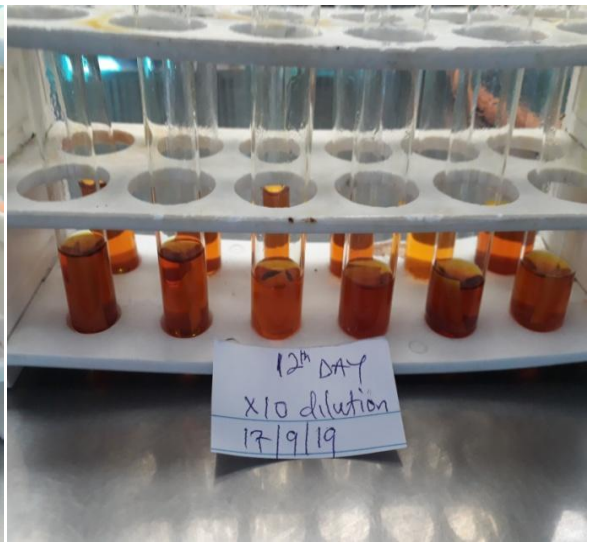
Presentations

1. Oral Paper presentation at an International Conference on Bioscience and Biotechnology (ICBB2019) titled "**SCREENING AND ENZYMATIC ASSAY OF ALKALINE FUNGAL CELLULASES"** held between 4th to 5th November 2019 organised by School of Bioscience and Biotechnology, LPU Phaghwara, Punjab India.
2. Poster Paper presentation at 60th Annual Conference of Association of Microbiologist of India (AMI2019) and International Symposium on Microbial Technologies in Sustainable Development of Energy, Environment, Agriculture and Health titled "**ISOLATION, SCREENING AND ENZYMATIC ASSAY OF ALKALINE CELLULASE PRODUCING FUNGI FROM SOIL OF KAPURTHALA DISTRICT, PUNJAB"** held 15th - 18th Nov. 2019 organized by Central University Haryana, Mahendergarh India.
3. Oral presentation of paper titled "**OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION OF ALKALINE CELLULASE ENZYME PRODUCED FROM *FUSARIUM OXYSPORUM* VSTPDKF2** at National conference on Microbial Bioprospecting: present and future scope held on 6th-7th March, 2020 Organized by Association of Microbiologist if India LPU unit at Lovely Professional University Punjab.

APPENDIX VI

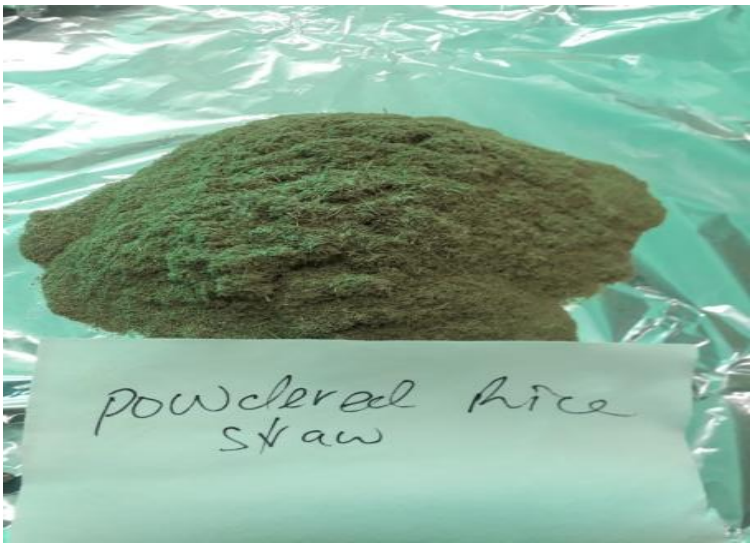
PICTURES OF SOME WORK DONE DURING THE RESEARCH

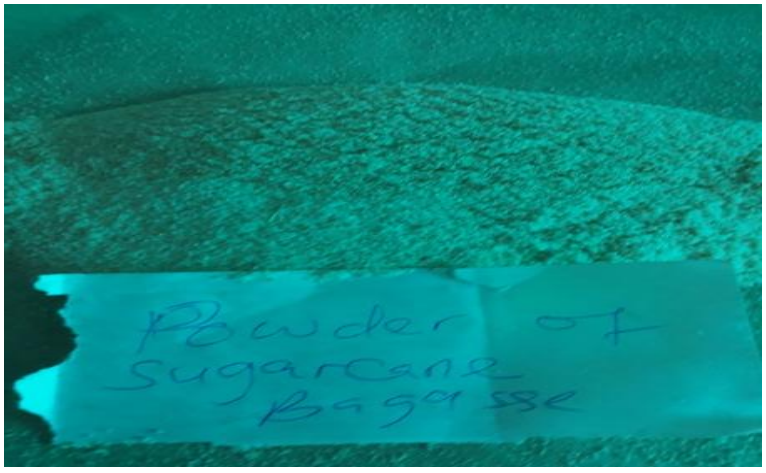




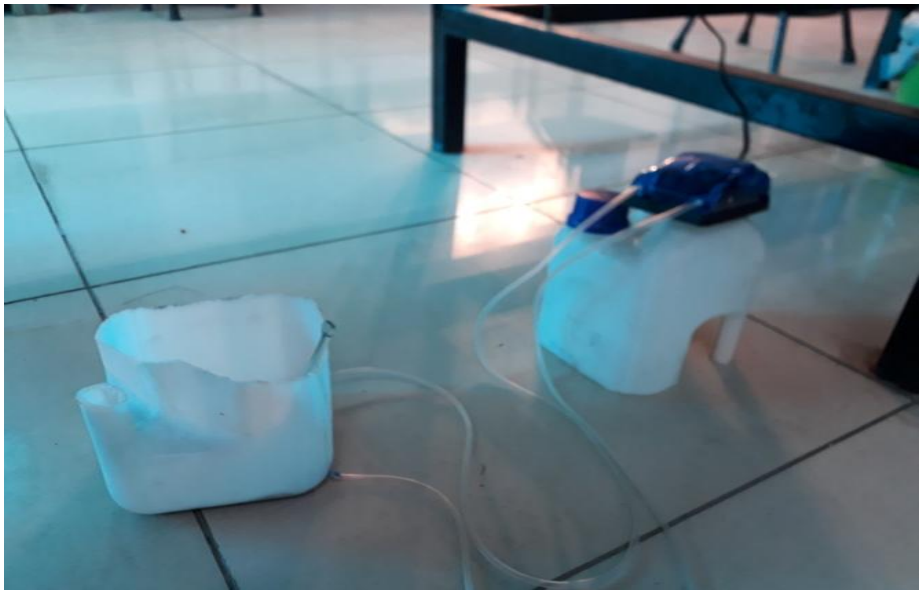
ENZYME ACTIVITY

CONGO RED





LIGNOCELLULOSIC WASTES



WASTE PAPER FLOTATION DEVICE