# INDUSTRIAL APPLICATION OF ALKALINE CELLULASE ENZYMES IN PULP AND PAPER RECYCLING: A REVIEW

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Industrial utilization of waste paper 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 the demand, due to global economic growth, an increasing number of trees are harvested each year, also leading to increased amounts of wastes and pollutants, which represent a serious hazard for the environment. Chemical agents, such as sodium hydroxide, hydrogen peroxide, sodium carbonate, diethylenetriaminepentacetic acid, sodium silicate and surfactants, are used in large quantities by paper industries as part of the conventional methods of deinking waste paper, leading to the need to apply expensive wastewater treatments in order to meet environmental regulations. On the other hand, enzymes, such as cellulase, lipase, xylanase, pectinase, hemicellulase, amylase and esterase, can substitute conventional chemical methods of deinking waste papers. These enzymes have been reported to be environmentally friendly, as compared to the chemicals involved in conventional methods. Several decades ago, it was established that microbial enzymes might be useful in the processing of paper, since it is composed of natural polymers, such as cellulose, hemicelluloses and lignin. However, despite their enormous potential, the industrial use of these enzymes is still limited, being affected by lack of microbial strains capable of generating a high amount of alkaline cellulase. This paper provides an insight into recent research performed with the objectives of optimizing alkaline cellulase enzymes production and applying them in pulp and paper processes.

***Keywords***: enzyme, alkaline, cellulase, industries

## INTRODUCTION

Cellulose is considered as one of the most abundant polysaccharides on earth, consisting mainly of D-glucose subunits connected by β-1,4-glycosidic bonds (Fig. 1). 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 catalyzes the hydrolysis process. Plant biomass is a term that refers to all organic materials found on earth, including lignocellulose, starch and sugar.[1] Among these, lignocellulose is composed of cellulose (50%), hemicelluloses (30%) and lignin (20%). 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.[2] Still, fungi are known to be the main organisms 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 the fermentation of these sugars to different products, is mainly carried out by bioconversion of lignocellulolytic materials.[3,4]

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.[5] As part of their natural ecological recycling, bacteria and fungi can break down lignocellulose with a complex collection of enzymes, which includes cellulase, hemicellulase and ligninase.

However, cellulase, which is an essential enzyme for bioconversion of cellulose to monosaccharides, is a family of three 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).[5] Endoglucanase (EG) targets internal O-glycosidic bonds spontaneously, resulting in glucan chains of different lengths; exoglucanase (CBH) 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 (Fig. 2). The microorganisms that produce these cellulase enzymes as they grow on cellulosic materials can be aerobic, anaerobic, alkaliphilic, mesophilic or thermophilic. Of these, the genera of *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Trichoderma*, *Fusarium*, *Mucor* and *Aspergillus* are the most extensively studied cellulase producers.[7]

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, which can grow only at pH values between pH 9 and above, and facultative alkaliphiles, which grow optimally at high alkaline conditions but also near neutral pH. Alkaliphilic microorganisms are an important source of useful, stable enzymes, including cellulases, hemicellulases, xylanase, esterase, pectinase and amylase.[8-12] They have a wide range of ecological niches, ranging from alkaline soda lakes[13] and soils[14,15] 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 springs (thermophiles), Antarctic seawater (psychrophiles), deep-sea hydrothermal vents (barophiles), alkaline soda lakes (alkalophiles), hot sulphuros springs (acidophiles) and natural/artificial salts (halophiles).[16] Alkaline cellulase has different biotechnological applications in industries ranging from paper, textiles, food, detergents and biofuels.[3] However, despite these enormous applications, the industrial uses of these enzymes are affected by lack of microbial strains capable of generating high amounts of alkaline cellulase.[16]

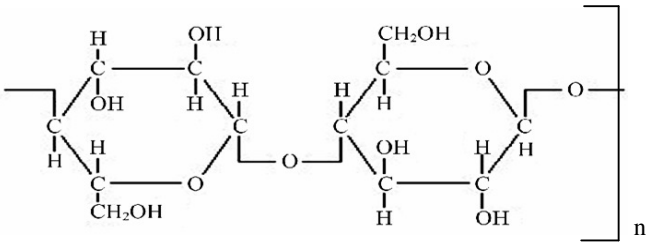


Figure 1: Structure of cellulose

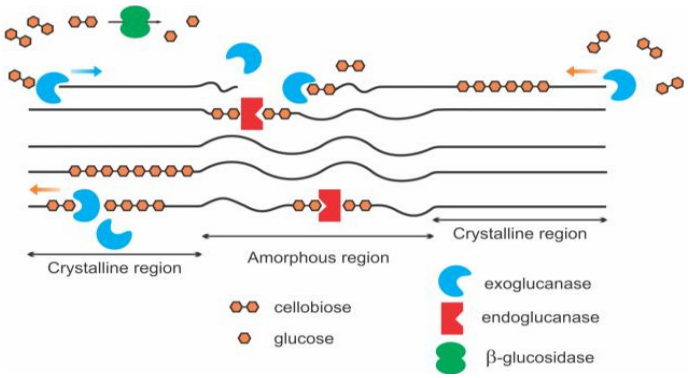


Figure 2: A simplified schematic representation of the enzymatic action of cellulase, involving exoglucanase, endoglucanase and β-glucosidase, on cellulose

Due to global economic growth, the demand for paper products has been constantly increasing. Industrial utilization of waste paper in the production of a new one has also been growing, which, on the one hand, means lower wood consumption by the pulp and paper industry, but,

on the other hand, involves more chemicals that reach the effluents and finally leach into the environment.[18] 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 part of conventional methods of deinking waste paper. To avoid environmental pollution, expensive wastewater treatments are then required to get the effluent to meet environmental regulations.[19] In this context, it has been found that enzymes, such as lipase, xylanase, pectinase, cellulase, hemicellulase, amylase, and esterase, can be used as an environmentally friendly method of deinking waste papers, to substitute chemical conventional methods. Although their potential was discovered several decades ago, microbial enzymes became commercially available and actually used in pulp and paper processing only in the previous decade, while at present microorganisms are also used in other industrial processing steps. 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.

In the last few decades, many studies have reported on the production of microbial cellulase enzymes and bioconversion of cellulose, especially for uses in paper industries. Presently, the fermentation conditions and the cost of enzyme production are the two main limiting factors of enzyme based bioconversion technology. Most of these researches usually compared the application of commercial cellulase enzymes for deinking purposes with the conventional deinking method and found a considerable improvement in the results obtained.[21,22] Given the importance of this subject, a comprehensive review of microbial alkaline cellulase enzymes, focusing on the optimization of their production and application in the pulp and paper industry, may provide an insight into the possibility of large scale production of these enzymes for commercial purposes.

## PRODUCTION OF MICROBIAL ENZYMES

Considering their many potential applications in different industries, such as pulp and paper, textiles, food, pharmaceuticals and biofuel, the production of microbial enzymes in general and cellulase enzyme in particular has been thoroughly investigated from a vast number of microorganisms (Table 1). Much research has focused on the production of cellulase from fungal species, isolated from various sources. Ramanathan *et al*.,[23] isolated *Fusarium oxysporum* from infected tomato plant parts for cellulase enzyme production using carboxymethyl cellulose as a source of carbon. From desert soil, Ahmed *et al.*[25] isolated and screened *Fusarium dimerum* and *Rhizopus oryzae* strains, capable of degrading cellulose, using CMC as a carbon source. Seeking exploitation of *Fusarium* sp. in commercial quantity for cellulase production, Dutta *et al.*[26] 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*.[27] In order to maximize enzyme production, nutritional and culture parameters affecting CMCase production were optimized. Other fungal isolates capable of producing cellulase, including *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium avenaceum* and *Cephalosporium acremonium*, were used for cellulase production using cellulose powder as a carbon source.[28] In related work, Remaz *et al*.,[29] isolated, screened and identified three fungi as *Aspergillus niger*, *Fusarium solani* and *Trichoderma viride*. These were found to possess the ability to degrade cellulolytic materials from their natural environment, namely, soil, tomatoes and orange samples in Khartoum and northern regions of Sudan.

The production of cellulases, in combination with other enzymes, was also investigated with the objective of enhancing productivity. Under solid state fermentation, cellulolytic and xylanolytic enzymes were produced from *Fusarium oxysporum* on corn stover, and their yield was enhanced by optimization of nutritional and physico-chemical parameters.[30] Xylanase was also produced from *Trichoderma viride* IR05 using solid substrate fermentation. Sugarcane bagasse was found as the best carbon source among different lignocellulolytic residues examined. The supplementation of xylose and tryptone as additional carbon sources, as well as of $NaNO_3$ and Tween 80, has been found efficient in improving xylanase production.[31]

Table 1
Cellulase enzyme produced by different microorganisms

| S/no. | Isolate | Enzyme | Maximum enzyme produced (U/mL) | Ref. |
|---|---|---|---|---|
| 1 | *Fusarium oxysporum* | Cellulase | CMCase 1.92; FPase 1.34 and β-glucosidase 1.78 | 21 |
| 2 | *Bacillus pumilus* 313SI | CMCase | 3.08 | 29 |
| 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* | 37 |
| 4 | *F. solani*, *F. oxysporum* and *F. chlamydosporum* | CMCase, FPase | CMCase (0.445) from *F. oxysporum* SF0801, FPase (9.25) from *F. oxysporum* SF1905 | 26 |
| 5 | *Trichoderma viride* IR05 | Xylanase | 72.4±1.42 U/g | 28 |
| 6 | *Fusarium oxysporum* VSTPDK | Endoglucanase | 3.62 U/mL | 31 |
| 7 | *Aspergillus hortai* | Endoglucanase | 0.23 | 24 |
| 8 | *Aspergillus niger* | Cellulase | 0.097 | 28 |
| 9 | *Aspergillus niger*, *Fusarium solani* and *Trichoderma viride* | Cellulase | 2.90 from *Aspergillus niger* | 26 |
| 10 | *Aspergillus niger* BCC14405 | Endoxylanase | 59.7 | 39 |
| 11 | *Aspergillus flavus* | Cellulase | 0.128 | 33 |
| 12 | *F. oxysporum* | Endoglucanase, Cellobiohydrolase, β-glucosidase, xylanase and β-xylosidase | Xylanase (1840), β-xylosidaze (0.041), CMCase (304), cellobiohydrolase (4.1) and β-glucosidase (0.140 U/g) | 27 |
| 13 | *Bacillus pumilus* 313SI | CMCase | 3.08 | 29 |
| 14 | *Bacillus* sp. | Amylase | 604.17 | 41 |
| 15 | *Enterococcus pseudoavium* | Amylase | 6.05 | 35 |
| 16 | Marine *Bacillus* VITRKHB | Cellulase | 7.80 | 43 |

Table 2
Cellulase enzymes obtained from different microorganisms at optimum pH and temperature

| Sample | Location | Organism | Enzyme | pH | Temp. (°C) | Ref. |
|---|---|---|---|---|---|---|
| Soil | Pulp and paper industries, India | *Bacillus subtilis* | Cellulase | 4.0 | 60 | [76] |
| Soil | Macuya rain forest, Pucallpa, Peru | *Aspergillus* sp. LM-HP32, *Penicillium* sp. LM-HP33 and 37 | Cellulase | 4.8-9.4 | 28 | [14] |
| Soil, compost, animal waste slurry | Jeju island, South Korea | *Bacillus subtilis* C5-16 and S52-2 | CMCase, avicelase, xylanase | 5.0 | 50 | [15] |
| Wild herbivore, rain deer | Wayanad, Kerala, India | *Escherichia coli* SD5 | Cellulase, xylanase | NA | 37-39 | [8] |
| Agricultural waste | Cairo, Egypt | *Bacillus thuringenesis* MAM-29, MAM-38 | Cellulase and xylanase | 3-7.6 | 60-80 | [9] |
| Soil | Iguazo rainfalls, Argentina | *Penicillium* sp. CR-313 and *Penicillium* sp. CR-316 | Cellulase | 4.5 | 65 | [52] |
| Soil | Punjab, India | *Fusarium oxysporum* VSTPDK | CMCase and FPase | 8 | 30 | [77] |

**Alkaliphilic bacterial strains**

The ability of microorganisms to break down cellulose is widely distributed among different bacteria and fungi. However, in order to be successfully applied in pulp and paper processes, the enzymes obtained must be stable and active at both high temperature and alkaline conditions. Among bacteria, a considerable number of Eubacteria like aerobic order *Actinomycetales* and anaerobic order *Clostridiales* possess cellulolytic ability. George *et al.*[32] isolated a novel alkalothermophilic actinomycete called *Thermomonospora*, with optimum growth between pH 9 and 50 °C from self-heating compost. The organism was able to produce a 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.[32] This strain named *Streptomyces thermoalkaliphilus* represents a novel species in the genus Streptomyces based on its phenotypic, chemotaxonomic and phylogenetic characteristics. Kalpana and Rajeswari[10] also reported *Streptomyces* isolated from agricultural waste, capable of producing enzymes for degrading xylan. *Streptomyces* spp. are a 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 samples of an alkaline soda lake in Maharastra, India, bacteria such as haloalkaliphilic *Marinobacter excellens*, *Alkalimonas delamerensis*, *Roseinatronobacter monicus* and *Rhodobaca bogoriensis* were identified for the first time in Lonar lake.[34] The bacterium described as *Heleococcum alkalinum* sp. was isolated on alkaline agar with carboxymethyl cellulose (CMC) and was the 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.*[35] The bacterium has the ability to grow in media with cellulose or cellobiose as the sole energy sources.

In various mangrove sites from Philippines, the conventional as well as the analytical profile index (API) was used to characterize and identify phenotypically five promising species of *Bacillus* producing cellulase enzyme, offering additional knowledge regarding the bacterial diversity of mangrove forests in the Philippines.[44] An old newspaper (ONP) waste was described as a carbon source for growing *Bacillus subtilis*, where avicelase and carboxymethylcellulase (CMCase) enzymes were estimated in the culture filtrate.

*Bacillus subtilis* CMCase has more activity at optimal temperature and pH than avicelase. Another bacterium called *Bacillus halodurans* was purified to homogeneity by Annamalai *et al.* and was reported to produce an extracellular haloalkaline cellulase by bioconversion of lignocellulosic waste.[45] This indicates that purified cellulase produced from *Bacillus halodurans* utilizing lignocellulosic biomass could be of great potential in industrial processes.

**Alkaliphilic fungal strains**

Just like bacterial enzymes, alkaline cellulase enzymes produced from alkaliphilic fungi are reported to have huge biotechnological applications in many industrial settings, such as textiles, paper, food, detergents and biofuels. However, their industrial applications have been hindered because of the lack of strains that can produce considerable amounts of enzymes. Over the past few decades, researchers have shown rising interest in cellulase production at alkaline pH. An extracellular alkali-stable endoglucanase from alkalotolerent *Fusarium* sp. was reported by Vyas and Lachke.[48] 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 additive to laundry detergent.[26] In addition, alkaline cellulase was reportedly obtained by Ravindran *et al*.[49] from alkalo-tolerant *Chaetomium* sp. isolated from mangrove leaves, using agricultural and industrial wastes as substrate, while Hmad *et al*.[51] produced alkaline cellulase from *Stachybotrys microspora*. In another research by Kladwang *et al*.,[16] about 490 alkaline tolerant fungi from a natural environment from different habitats in Thailand were identified using Petri dishes containing potato dextrose agar medium buffered at pH 11.0.

Soil is one of the most 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 with the highest productivities were found to be the *Penicillium* sp. LM-HP33, *Penicillium* sp. LM-HP37, as well as *Aspergillius* sp. LM-HP32. These fungal strains have been determined to be suitable for the production of alkaline cellulase.[14] High cellulase activity was found by Picart *et al*.[52] from a fungal strain in subtropical soils, having a medium supplemented with rice straw. Crude cellulase produced by *Penicillium* sp. CR-316 has potential in industrial applications, since it shows activity and stability at high temperature and produces a thermostable cellulase. Bilanenko *et al*.[53] also reported an isolate representing an Ascomycete group from saline soda soils of Central Asia and Africa.

**OPTIMIZATION OF MICROBIAL ENZYMES PRODUCTION AND APPLICATION IN PAPER INDUSTRY**

Microbial cellulases have been focused on as important biocatalysts, being multiplex in nature and bearing extensive applications. Cellulase and hemicellulase enzymes are both synthesized by fungi and bacteria. As compared to fungi, bacteria have a higher rate of cellulase enzyme production, due to their advantage of higher growth rate. Medium composition and fermentation conditions were reported to influence the production of cellulase by microorganisms and thus are considered as significant factors for optimization. Namely, physical parameters, such as pH, temperature, and incubation time, as well as nutritional factors, like carbon and nitrogen sources, are the major factors affecting cellulase production.[54]

Several efforts have been made for achieving high production of cellulase by determining the best possible fermentation conditions. The one-factor-at-a-time (OFAT) approach, which is time-consuming and very expensive, is one of the classical methods usually applied for this process. The combinations of interactions between physical and nutritional parameters for the production of cellulase can be numerous and thus it is difficult to estimate their significance through this one-factor-at-a-time approach. Therefore, effective statistical and experimental design procedures have been developed. A collection of statistical techniques, called Response Surface Methodology (RSM), used for designing experiments, as well as evaluating the effects of parameters for optimum production, has been reported for optimization of cellulase enzymes.[55] Contrary to conventional techniques, statistical tools such as RSM have gained considerable attention due to their easiness modeling for different microorganisms, considering the applicability of enzymes in many industrial processes. Aanchal *et al*.[56] 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 concentrations, 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 samples of *Lantana camera.* Under optimized conditions, a 5.35-fold increase and a 6.62-fold increase were reported for CMCase and FPase, respectively.[8]

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.[57] They identified temperature, pH, substrate concentration, inducer concentration, inoculum age and agitation speed as the most important parameters to optimize for the production of cellulase. The same *Trichoderma reesi* RUTC30 was also used for the optimization of cellulase production using sugarcane bagasse as a carbon source by Mekala *et al*.[58] The research reported to have optimized parameters such as temperature, incubation time and inducer concentration, using the Box-Behnken experimental Design (BBD). The result indicated the highest FPase production of 25.6 U/gds when inducer concentration was 0.33 mL/gds, temperature – 33 °C and incubation time – 67 h. Saravanan *et al*.[59] revealed an increase of cellulase production of 9.23 U/mL and 6.98 U/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, inoculum concentration and incubation time. Their results proved that BBD is a more efficient statistical tool for optimizing enzyme production, as compared to genetic algorithm.

Several microbial enzymes, used either individually or in combination, have been found to be applicable in the removal of ink from waste paper. Enzymes, such as cellulase, hemicellulase, α-amylase, lipase, xylanase and other ligninolytic enzymes, are efficiently used in deinking processes (Table 3). The enzymatic treatment is favorable to deinking as enzymes enable ink detachment without any discharge of harmful chemicals, thus rendering the process eco-friendly. Studies reported on deinking of mixed office waste consisting of photocopied paper, using a commercially available enzyme.[72] Using carboxymethylcellulose as substrate, Ariffin *et al*.[73] produced cellulase enzyme from a local isolate of *Bacillus pumilus* EB3. This enzyme was purified using ion exchange chromatography and characterized. Rawat and Tewari[74] isolated and identified a *Bacillus subtilis* strain LFS3, which hydrolyzed carboxymethylcellulose (CMC). Gel filtration chromatography, ion exchange and sodium sulphate precipitation were the methods used to isolate and screen the cellulase enzyme, with an overall recovery of 15%. The optimum temperature and pH for achieving an active profile of this enzyme were 60 °C and 4.0, respectively.

Another study was conducted to optimize cellulase production from a versatile *Aspergillus fumigatus fresenius* (AMA), targeting its application in efficient deinking and enzymatic hydrolysis of Solka-Floc and bagasse, by the Box-Behnken Design (BBD) of experiments for RSM. The CMCase obtained proved capable of removing 53% residual ink and increased the brightness of handsheets by 4.32% ISO.[60] Response surface methodology (RSM) was used as a statistical tool through BBD to optimize the moisture content, pH, temperature and incubation time, which allowed producing maximum cellulase and pectinase. A significant increase in enzyme productions was remarked as compared to the conventional one-factor-at-a-time approach.[54] The 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).[56] A newly isolated *Aspergillus niger* HN-1 was also used for the production and optimization of cellulase by Plackett-Burman and CCD statistical models. The design expert software was capable of improving FPase and β-glucosidase activities by 2 and 3 fold increases, respectively.[57]

Table 3
Microbial enzymes and their applications in pulp and paper industry

| S/no. | Microorganism | Enzymes | Application/Effects | Ref. |
|---|---|---|---|---|
| 1 | Commercial enzyme | Cellulase | Ink removal, reduction of drainage time | 18 |
| 2 | *Trichoderma harzianum* | Cellulase and xylanase | Reduced drainage time, high deinking efficiency and pulp brightness | 65 |
| 3 | Commercial enzyme | Cellulase | Not good in terms of specks surface of deinked paper | 21 |
| 4 | Commercial enzyme | Cellulase | Detached significant amounts of ink from ONP/OMG | 22 |
| 5 | *Aspergillus niger* | Cellulase and hemicellulase | Enhanced deinking efficiency | 67 |
| 6 | *Streptomyces* sp. L22001 | Xylanase | Biobleaching effect | 68 |
| 7 | *Bacillus altitudinis* | Xylanase | Potential for biodeinking and biobleaching | 11 |
| 8 | *Bacillus* sp. CKBxID | Xylanase | Deinking agent for recycled waste paper | 69 |
| 9 | *Alkalothermotolerant* | Xylanase and pectinase | Commercially viable, yielding better paper quality | 70 |
| 10 | *Aspergillus niger* | Xylanase | Deinked old newspaper with improved brightness, removal of surface ink particles from ONP pulp | 71 |
| 11 | *Aspergillus nidulans* | Xylanase | Ink removal, increased brightness of recycled paper | 72 |
| 12 | Commercial enzyme | Laccase | Reduction of lignin content, useful in the process of bio-pulping | 73 |
| 13 | *Enterococcus pseudoviun* | Amylase | Effectively deinked and decolorized paper pulp within four days of incubation | 42 |
| 14 | Commercial enzyme | Laccase and hemicellulase | Deinked old newspaper | 74 |
| 15 | Commercial enzyme | Cutinase and amylase | Increased pulp brightness and ink removal | 12 |
| 16 | *Mocur circinelloides* WSSDB2F1 | Cellulase | High ISO brightness, tensile, burst and tearing strength, as compared to chemical deinking | 69 |

Three filamentous fungi, *i.e. Mucor indicus*, *M. hiemalis* and *Rhizopus oryzae*, were also investigated for cellulase production by solid state fermentation using wheat bran as sole carbon source. RSM was used to optimize fermentation parameters, including temperature, incubation period and moisture content, to achieve maximum production of cellulase.[37]

The production of cellulase enzyme by *Bacillus pumilus* EWBCM1 was optimized using RSM based on the Central Composite Design, by varying parameters such as galactose and malt extract contents, as well as incubation time.[43] This statistical software was also used for the optimization of other enzymes for industrial applications, produced from different microorganisms. Lipase isolated from *Aspergillus niger* strain AC-54 was optimized initially through Plackett-Burman (PB) design, followed by CCD. The predicted activity of this enzyme from the statistical model was validated and confirmed by experimental results.[58] From slaughterhouse polluted water, a keratinolytic enzyme producing bacterium was isolated and identified as *Bacillus pumilus* A1. Placket-Burman was initially applied to identify the best culture medium ingredients and conditions for maximum production of keratinase. The optimization of five important parameters, namely the contents of feather meal, soy peptone, NaCl, KCl and $KH_2PO_4$, was conducted by Central Composite Design, which generated a 3.4-fold increase in keratinase production, as compared to the optimization by Placket-Burman.[64] In similar research, the full factorial design and the central composite design were applied to evaluate the effects of major amylase production parameters isolated from *Bacillus* sp. under submerged fermentation.[34] Khonzue *et al*.[39] used response surface methodology as a statistical tool for the optimization of fermentation parameters to obtain endoxylanase from *Aspergillus niger* BCC14405. They also investigated the potential application of crude endoxylanase for biobleaching of eucalyptus pulp. The enzyme was able to increase pulp brightness and viscosity, suggesting an increase in its cellulose content.

A fungus *Coprinopsis cinerea* was found to have the ability to produce cellulase and xylanase enzymes, with high potential in deinking photocopier waste paper, as reported by Pathak *et al*.[65] They investigated the enzyme dose, point of enzyme addition, pulp consistency and reaction time necessary to achieve the maximum possible deinking efficiency, without affecting the strength properties of paper. Their results confirmed the ability of crude enzyme produced by *C. cinerea* in deinking of photocopier waste papers.

The effects of using cellulase for deinking office waste paper were investigated by Tsatsis *et al*.[21] Active enzymes in the deinking experiment led to better deinking results. It was discovered that the use of enzyme had a disadvantage when considering the specks surface of deinked paper sheets, as compared to conventional deinking. They suggested that more research is needed to develop formulations of enzymes, with better performance under alkaline conditions, to address different types of printed paper (photocopied and laser printed).

Abo-State *et al*.[9] isolated a *Bacillus* strain from agricultural waste and identified it as *Bacillus thuringenesis*, with the ability to produce cellulase and xylanase at suitable pH and temperature. The stability of the enzyme at different temperatures (60-80 °C), for different duration, was also investigated. Zhang *et al*.[22] evaluated three commercial cellulase enzymes for their application in 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 3 h. Despite the fact that cellulase enzyme was able to remove a significant amount of ink from ONP/PMG, it had lower efficiency than when using conventional methods based on either sulphite or alkaline deinking chemistry. Meanwhile, none of the three cellulase enzymes tested were able to separately deink aged ONP/OMG, and poor deinkability was also observed by using either sulphite or alkaline chemistry. However, the research indicated a significant increase in deinking efficiency when a combination enzyme and sulphite was applied, revealing a potential strategy for achieving effective deinking of old newspapers at neutral pH.

In an effort to increase the enzyme production rate, fungal cellulase has been pursued by several mills with the objective of improving pulp drainage. The enzyme was also used in the production of easily biodegradable products, including paper towels and sanitary paper.[77] The laccase mediator system was used in a study conducted to compare the effects of the application of cellulase/hemicellulase for deinking pulps originating from newspapers and magazines. In this regard, commercially available

endoglucanase and endoxylanase and a commercial laccase were evaluated in the presence of synthetic or natural mediators. The researchers concluded that there a number of other factors to be considered in the application of enzymatic deinking processes, in addition to those related strictly to the enzymes, and these include the used ink types, printing methods and fibre/ink separation process.[72] Lee *et al.*[67] also developed a laboratory procedure for enzymatic deinking of waste papers using cellulase and hemicellulase enzymes produced from *Aspergillus niger*. Using an optimized flotation system at 6.0 pH and 45 °C temperature, deinking efficiency using these enzymes was enhanced to 95%. The deinked papers were found to have similar properties to those of commercial papers, indicating the effectiveness of the developed enzymatic process.

Overall, enzymatic deinking has a number of advantages over conventional deinking, as it reduces alkali usage, improves fibre brightness and fibre strength properties. Moreover, the enzymatic deinking process also prevents alkaline yellowing of the pulp and has a lower environmental impact.

## CONCLUSION

The major disadvantages of using conventional deinking methods consist in the use of hazardous chemicals, which have negative effects on the environment, and involve high costs for treating effluent wastewater to avoid pollution. On the other hand, although there are different commercially available enzymes as replacement to chemical deinking, most of these enzymes are quite expensive. The method of on-site cellulase enzyme production from bacteria and fungi is both cheaper and eco-friendly. It also decreases the significant amount of chemicals necessary to reach high pulp brightness in bleaching processes. Also, there have been reports of combining chemical deinking with the enzymatic method, targeting higher efficiency. Thus, considering the physicochemical conditions of chemical deinking, as well as those of other pulp and paper processes, involving high pH and temperature, the isolation of thermoalkaliphilic microorganisms that can produce considerable amounts of cellulase enzymes for deinking purposes is viewed as of paramount importance. Since in earlier research, most microorganisms capable of producing enzymes, especially fungi, that have been focused on normally grow in acidic to neutral pH, and little research has been reported on alkaline cellulase enzymes, especially on their applicability in the pulp and paper industries, this review focuses on this less researched, but important area.

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