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Screening, Isolation and Partial Characterization of Cellulase from Cellulase Producing Bacteria for Industrial Applications

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Abstract

Microbial cellulases have demonstrated their potential use across several industries such as pulp and paper, textiles, laundry, biofuel production, food and feed sectors, brewing, and agriculture. Owing to the intricate nature of enzyme systems and their significant industrial potential, cellulases have emerged as a promising subject for investigation by both academic and industrial research entities. The cellulase producing microorganism is cultured in large quantities for the enzyme production. The major source of cellulase producing microorganisms are bacteria and fungi. The obtained carotenoids have the potential to be used as colouring agents in the food and beverage industries. So currently, considerable focus has been dedicated to the existing understanding of cellulase production and the hurdles in cellulase research, particularly regarding the enhancement of process economics across different industries. This paper examines scientific and technological advancements as well as future applications of cellulases across various industries.

Keywords

Cellulase Enzyme, Isolation and Screening of Cellulolytic Microorganisms, Lignocellulosic biomass, Microorganisms.

INTRODUCTION

Carbohydrates represent the largest category of organic compounds present in living beings. Carbohydrates act as energy sources and vital structural elements in organisms, as well as being a component of nucleic acid structures. Carbohydrates are classified into four categories: monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Polysaccharides are sizable compounds that can comprise up to 10,000 linked monosaccharide units. Polysaccharides make up the majority of the structural and energy-storage carbohydrates present in nature. Cellulose and starch are the most typical examples of polysaccharides. Cellulose is a straight-chain polymer made up of glucose units connected by β-1, 4 glycosidic bonds. It is the most plentiful polymer globally and a key part of plant biomass. It offers stability to the plants and exists as microfibrils in the plants that create the structurally robust framework in the plant cell wall. Cellulose is devoid of smell and taste, insoluble in water and many organic solvents, and is resistant to breakdown. Plants produce 4×10^9 tons of cellulose each year. The cellulose chains are linked by van der Waals forces and both intra- and intermolecular hydrogen bonds. The individual stabilizing elements may be weak, yet together they create strong connections. Cellulose serves as a crucial carbon source on Earth, with its biosynthesis rate estimated at 0.85×10^11 tons each year by terrestrial and aquatic plants. Agricultural byproducts serve as a significant source of lignocellulosic biomass, comprising hemicelluloses (20-30%), cellulose (30-40%), and lignin (20-30%). The waste products from agriculture consist of 70% moisture and 30% solid content [1-25].

Since these waste materials can lead to environmental pollution, it is crucial to biologically decompose the waste product. These waste materials are biologically decomposed for both economic and environmental reasons, serving as an alternative method for managing agricultural, industrial, and hazardous wastes. Enzymes are referred to as biocatalysts and are essential in metabolic and biochemical processes. Microbial enzymes are preferred due to their active and stable characteristics compared to enzymes from plants and animals. In difficult conditions, the microorganisms cannot develop and generate the enzymes. Recent research focuses on isolating bacterial and fungal strains from extreme conditions like high temperatures and pH, salinity, heavy metal presence, and organic solvents to achieve a high yield of enzymes. Cellulase comprises a collection of hydrolyzing enzymes that significantly contribute to the biodegradation of cellulosic waste, thereby diminishing environmental pollution. This leads to the creation of important substances such as energy fuels and alternative feed sources.

Cellulase breaks down the β-1, 4 glycosidic bonds of cellulose. The multienzyme system breaks down cellulose via the cooperative actions of three enzymes: endo-β-1,4-glucanase, β-D-glucosidase, and cellobiohydrolase. Cellulase enzyme finds extensive application in the textile sector, wine and brewing industries, cleaning products, food manufacturing, and the paper and pulp sector. Microbial enzymes are manufactured on an industrial scale through surface or submerged fermentation methods. The microorganism that produces cellulase is grown in substantial amounts for enzyme production. The primary sources of microorganisms that produce cellulase

include bacteria (aerobic, anaerobic, thermophilic, and mesophilic) as well as fungi. Bacteria are utilized more frequently than fungi due to their faster growth rate in comparison to fungi.

Cellulase is generated by bacteria such as Cellulomonas, Cellvibrio, Pseudomonas, Bacillus, and Micrococcus. It is generated by fungi such as Trichoderma, Aspergillus, and Penicillium species. The molecular docking technique has been utilized to investigate the process of biodegradation. It's an effective way to comprehend the relationship between the enzyme and the organic contaminant. This technique is employed to assess if the enzyme can degrade the organic pollutant. This is accomplished by determining if the organic pollutant can fit into the enzyme's active site based on the steric hindrance of the residues and the characteristics of the active site pocket. The goals of the current research are

- To identify and evaluate cellulase-producing microbes from soil.
- To define the isolated bacteria through morphological and biochemical examination.
- To perform the Batch fermentation process.
- To enhance the circumstances that promote enzyme production.
- To validate the cellulose degrading effectiveness of the generated enzyme using paper pulp.
- To analyze the digested paper pulp through FTIR characterization.
- To perform the quantitative analysis of the enzyme using the DNS method.
- To conduct molecular docking to examine the interactions between the enzyme and the contaminants.

REVIEW OF LITERATURE

Cellulase Enzyme

Cellulase is a multifaceted enzyme system that includes endo-1, 4-β-D-glucanase (EC-3.2.1.4), exo-1, 4-β-glucanase (exocellobiohydrolase, EC-3.2.1.91), and β-D-glucosidase (β-D-glucosideglucanohydrolase, EC-3.2.1.21). The combined activity of cellulases breaks down β-1, 4 bonds in cellulose chains. In contrast to other glycoside hydrolases, cellulases can cleave β-1,4-glucosidic bonds linking glucosyl units. The enzymatic cleavage of the β-1, 4-glucosidic linkages in cellulose occurs via an acid hydrolysis mechanism. The products of hydrolysis can either lead to the retention or inversion of the anomeric configuration at carbon-1 on the reducing end. Endoglucanases, also known as carboxymethylcellulases (CMCase), randomly cleave the glycosidic bonds in the amorphous areas of cellulose. This produces long-chain oligomers. These long-chain oligosaccharides are broken down into short-chain oligosaccharides through the activity of exoglucanases or cellobiohydrolases. Through the processive action of exoglucanases on reducing or nonreducing ends, cellobioseor glucose is released. The cellobiohydrolases (CBH) are the exoglucanases that have been researched the most. Different microorganisms produce them. Cellobiose acts as an inhibitor for endoglucanase and cellobiohydrolases. β-Glucosidases (BG) break down cellobiose and soluble cellodextrins into glucose [10-20].

Collaboration is essential for thorough hydrolysis of cellulosic substances. There are four types of synergisms:

- Synergy between endoglucanases and exoglucanases in endo–exo interactions,
- Exo–exo interaction among exoglucanases,
- Collaboration between exoglucanases and β-glucosidases, and
- Synergistic interactions within catalytic domains and carbohydrate-binding modules (CBMs).

MICROORGANISMS IN CELLULASE PRODUCTION

Acquiring enzymes economically is a crucial aspect of industrial biotechnology. Among various cost-effective approaches for cellulase production, submerged fermentation (SmF) and solid-state fermentation (SSF) employing microorganisms are the most widely used methods. Many microorganisms can produce cellulase. Fungi are the primary microorganisms that produce the cellulase enzyme. Other microorganisms such as bacteria and actinomycetes are also known to generate cellulase. Similar to other enzymes, advancements in molecular biology significantly propelled cellulase research as an academic field during the 1980s. The idea of cloning and expressing a cellulase gene in a different microbe was successfully achieved in 1982–83. Currently, the researchers are working on improving technologies for enzyme reuse, which involves utilizing carrier-free biocatalysts and magnetic biocatalysts. Fungi generate cellulase solely when cellulose is available, while bacteria continuously produce cellulase. Various approaches to enhance enzyme production involve optimizing fermentation parameters, making alterations in the genome, developing new strains, and isolating from diverse sources, among others. Additionally, bacteria from oceanic settings are being isolated to acquire enzymes that exhibit greater tolerance to salt, pressure, and temperature, which are beneficial for applications across diverse industries. The idea of cloning and expressing a cellulase gene in a different microbe was successfully achieved in 1982–83 [15-25].

Fungi in Cellulase Production

Fungi possess long hyphae that can penetrate deeply into cellulosic substances, allowing them to access greater amounts of cellulose. Fungi generate a greater quantity of cellulase in comparison to other cellulase-producing organisms. Trichodermareesei is the most commonly utilized fungal strain for cellulase production and is regarded as the model fungus for exploring enzyme production. Additional fungi like Humicola, Trichoderma, Penicillium, and Aspergillus are likewise utilized for increased yield. The cellulase system of T. reesei (teleomorph: Hypocreajecorina,

formerly known as Trichodermaviride) has been a research focal point for 50 years. T. reesei generates a minimum of two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV), and two β-glucosidases (BGLI and BGLII).

Bacteria in Cellulase Production

Numerous bacteria, both aerobic and anaerobic, generate cellulase enzyme. While fungi are primarily utilized for producing cellulase, bacteria are favored in certain situations, particularly for recombinant enzymes. Bacteria exist in nearly all environments and grow faster than fungi. Extremophiles can thrive in severe environments, making them simpler to cultivate. Aerobic and anaerobic bacteria employ distinct cellulolytic strategies. Anaerobes possess a sophisticated cellulase system. The cellulases of most anaerobes are found on their cell surface or within the glycocalyx matrix. A limited quantity of extracellular cellulase is secreted into the medium. While aerobic bacteria break down cellulose by releasing a significant quantity of extracellular cellulase into the culture medium, certain strains also possess cellulase complexes on their cell surfaces. A researcher isolated a bacterium with 98% similarity to Bacillus cereus from a fish gut and fine-tuned the conditions for cellulase production. A temperature of 30 °C, pH 9.0, and an inoculum size of 2% (v/v) were optimal for exoglucanase production during submerged fermentation. The highest cellulase production occurred at 0.09% MgSO4, 0.5% yeast extract, and 0.03% peptone. The researcher investigated the cellulolytic and amylolytic capabilities of the bacterial strains Bacillus megaterium and Bacillus mycoides. The findings of this research indicated that they can serve as probiotics in feeds for fish and shrimp.

ISOLATION AND SCREENING OF CELLULOLYTIC MICROORGANISMS

Nikky extracted cellulolytic Pseudomonas sp from soil at a waste disposal site. The isolate demonstrated peak activity at pH-7.0 and 50°C. The enzyme activity tripled with manganese ions present, likely serving as a cofactor for enhanced activity. To lower the production expenses of cellulase, two new fungi that generate cellulase (Aspergillusniger and Trichoderma sp.) were isolated from 4-5% (w/v) municipal solid waste residue. A mixture of 1.0% (w/v) peptone and yeast extract was identified as the optimal combination for cellulase production by Aspergillusniger and Trichoderma sp. The ideal temperature and pH for cellulase production by A. niger were 40°C and 6-7, while for Trichoderma sp., they were 45°C and 6.5. The production of cellulase from Aspergillusniger and Trichoderma sp. offers an advantage, as the enzyme output is typically greater than that of other fungi.

Bacteria that produce cellulase were isolated from soil, with Pseudomonas fluorescens identified as the most effective cellulase producer.

The ideal conditions for cellulase production were 40oC and pH-10, using glucose as the carbon source and ammonium sulfate as the nitrogen source. Coconut cake was discovered to enhance cellulase production. The researcher extracted cellulase-producing Paenibacillussp from molasses. The isolated strain Paenibacillussp exhibited peak cellulase activity at pH 7.0 and 40°C following 24 hours of incubation with a 1% CMC substrate concentration.

Table 1. Microorganisms used for cellulase production

Group	Organism	Reference
Fungi	*Aspergillusaculeatus* *A. fumigatus* *A. Niger* *cellulyticum* *Fusariummoneliforme* *Humicolainsolens* *Myceliophthorathermophila* *Neocallimastix* *Penicilliumbrasilianum* *P. chrysogenum*	29-35
Bacteria	*Acetobacterxylinum* *Bacillus cereus* *B. lautus* *B. mycoides* *B. subtilis* *Caldocellumsaccharolyticum* *Clostridium acetobutylium* *C. straminisolvens* *C. thermocellum*	12-20
Actinomycetes	*Cellulomonasfimi* *C.bioazotea* *C.uda* *Streptomyces drozdowiczii*	22-25

Prabesh identified microorganisms that produce cellulase from municipal solid waste and rice straw waste. Isolates of Bacillus and Pseudomonas demonstrated peak activity at 37°C, whereas Serratiasp exhibited highest cellulase activity at 35°C. Shaikh gathered soil samples from various locations, including garden soil, wood furnishing areas, and a sugar cane farm. Additionally, water samples were obtained from paper industry waste and municipal waste, leading to the isolation of 14 cellulase-producing microorganisms.

The ideal conditions for cellulase production by Pseudomonas sp were found to be 40°C and pH 7 after 96 hours of incubation. The cellulase enzyme derived from Bacillus sp exhibited peak activity at 50oC and pH-7.5 following 96 hours of incubation. The cellulose-producing Bacillus pseudomycoides and Bacillus massilioanrexius were extracted from sugarcane bagasse. The highest enzyme concentration was reached at pH-7.0 (4.7 mg/ml) and 40°C after 72 hours of incubation (4.25 mg/ml).[26] extracted cellulase-producing Bacillus sp 313SI from soil samples collected from various environments, including sugarcane fields, rice fields, the paper industry, cattle sheds, decayed

fruits and vegetables, and cow dung samples. Under agitation, Bacillus sp. 313SI exhibited peak activity at pH 8, 30°C with 0.75% substrate concentration and 0.3% inoculum concentration following 48 hours of incubation. At pH-8 and 35°C, it exhibited peak activity with 1% substrate concentration and 1% inoculum after 60 hours of incubation in stationary conditions.

DIFFERENT SUBSTRATES USED FOR ENZYME PRODUCTION [17] [18]

LignocellulosicBiomass

Lignocellulosic biomass, which consists of forestry, agricultural, and agro-industrial waste, is a plentiful, renewable, and low-cost energy source. It includes diverse materials such as sawdust, poplar, sugarcane bagasse, waste paper, brewer's grains, switchgrass, and residues like stems, stalks, leaves, husks, shells, and peels from cereals including rice, wheat, corn, sorghum, and barley. Lignocellulosic waste builds up annually in significant amounts, resulting in greater environmental issues. Thus, in addition to the environmental issues resulting from their accumulation in nature, not utilizing these materials represents a loss of a potentially valuable resource (Mussatto and Teixeira, 2010)

Table 2. Composition of various lignocellulosic biomass

Agricultural& HerbaceousBiomass	Lignin(%)	HemiCellulose(%)	Cellulose(%)	Ash(%)	Reference
Wheat straw	17-19	26-32	33-38	3.74	19-35
Rice straw	12-14	23-28	28-36	19.8	
Barley straw	14-19	27-28	31-45	-	
Rye straw	16-19	27-30	33-35	-	
Oat straw	16-19	27-38	31-37	-	
Rice husk	26-31	18-21	25-35	17.27	
Sugarcane bagasse	20-42	19-25	42-48	-	
Sweet sorghum bagasse	14-21	18-27	34-45	-	
Corn stover	7-19	24-26	38-40	6-8	
Corn cobs	14-15	35-39	42-45	3.53	
Bamboo	20.81	19.49	39.80	1.21	
Switch grass	10-40	30-50	5-20	5-6	
Hazelnut shell	42.1	28.2	25.2	1.4	

CarboxymethylCellulose (CMC)

Carboxymethyl cellulose (CMC) is a anionic, water-soluble form of cellulose. Soluble cellulose derivative is simply degraded by microorganisms and CMC can be used to assay cellulase. The extracellular cellulolytic activity is commonly discovered on the agar media plate consists of carboxymethyl cellulose (CMC) as substrate.

Effect of Different Substrates on CellulaseProduction

The microorganism Streptomyces diastaticus PA01, which produces cellulase, successfully grew and generated endoglucanase utilizing oat bran (OB) or sugarcane bagasse (SCB) along with corn steep liquor (CSL) as carbon and nitrogen sources, respectively. Bacillus species producing thermophiliccellulase was extracted from cow dung. The peak enzyme activity occurred with carboxymethyl cellulose as the carbon source, peptone as the nitrogen source, at pH-7 and a temperature of 51°C. Cellulase production by Aspergillusniger ITBCCL74 grown on alkali-treated rice straw through solid-state fermentation methods. Rice straw is one of the most plentiful lignocellulosic waste materials globally. A high cellulose activity of 3.12 U/ml was achieved when the fungus was grown on a substrate with 75% moisture, at pH-4, for 4 days at 60oC and a substrate concentration of 1.25% (SitiMaftukhah and Abdullah, 2018). Optimization of solid state fermentation using raw oil palm frond leaves (OPFL) as the substrate for producing extracellular cellulase by the novel Rhizopusoryzae UC2 was conducted. The ideal conditions for solid state fermentation (30°C, 40% moisture, 2.0x10^8 spores/g inoculum) produced the highest carboxymethylcellulase (CMCase) activity (94.68 U/g), exhibiting a wide pH spectrum from 6.0 to 12.0. The enzyme complex exhibited peak activity at an acidic pH and temperature levels between pH 3.0-5.0 and 50-60°C. Trichodermasp obtained from olive stones was cultivated on food production wastes. All three types of cellulase were identified in the culture supernatant and confirmed through spectrometry analysis.

PRODUCTION OF CELLULASE UNDER SOLID STATE FERMENTATION

Solid state fermentation is an appealing method for producing cellulase and xylanase affordably because of its reduced capital costs and lower operational expenses.

Selecting an inexpensive and readily accessible substrate seems crucial for lowering the enzyme cost. Fermentation in solid-state employs solid materials like bran, bagasse, paddy straw, various agricultural byproducts, and paper pulp. The primary benefit of utilizing these substrates is that nutrient-dense waste materials can be effortlessly repurposed as more economical substrates. Cellulose-degrading filamentous fungus Talaromycesstipitatus MTCC 12687 was utilized for cellulase production through solid state fermentation. Various lignocellulosic materials such as wheat bran, wheat straw, rice straw, corncob, sugarcane tops, and carrot grass served as carbon sources in solid-state fermentation. Prabhjot Kaur stated that the microbial strain Bacillus subtilis PJK6 could generate a significant cellulase yield using wheat bran as the exclusive carbon source under solid-state fermentation at moderately elevated temperatures, showing nearly equal activity at pH levels 6 and 9 at 37°C. The three filamentous fungi producing cellulase, Mucorindicus (163 U/g), Mucorhiemalis (188 U/g), and Rhizopusoryzae (281 U/g), exhibited the highest cellulase activities when dry wheat bran served as the substrate. The ideal temperature for all fungi was 26.6oC, while the best moisture content and cultivation duration for M.indicus and R.hiemalis were 71.8% and 33.2 hours, respectively. The reported parameters for M.hiemalis were 38.18% and 66.81 hours **[24-27]**.

The highest cellulase activity generated by the isolated Aspergillusterreus RS2 was 124.94 U/g dry substrate. This was accomplished under the best conditions, as detailed: 3.75 g (1.5% w/v) of rice straw was hydrated with 11.25 ml of modified Mandel's medium at pH-7, incubated at 30°C for 8 days. The enzyme's activity in breaking down rice straw showed the release of reducing sugars at a rate of 343.98 mg/g of dry substrate, with a saccharification percentage of 34.4% noted after 4 hours of hydrolysis. Bharti Amit Kumar observed peak endoglucanase activity (26.33 IU/gds) and β-glucosidase activity (34.30 IU/gds) from Aspergillusniger NFCCI4113 when utilizing wheat bran as a substrate during solid state fermentation at 30°C, pH-7.0, with 70% moisture, and peptone (1.0% w/v) as the nitrogen source after an incubation period of 6 days.

PRODUCTION OF CELLULASE UNDER SUBMERGED FERMENTATION

Submerged fermentation (SmF) employs liquid substrates that flow freely, like broth and molasses (Subramaniyam and Vimala, 2012). This fermentation method is most appropriate for microorganisms, like bacteria, that need elevated moisture levels. The key stages in the production of cellulase through submerged fermentation include the following:

- Choice of effective strain as cellulose provider.
- Choosing waste as a substrate, such as lignocellulosic agricultural residuals.
- Increasing carbon content in lignocellulosic waste through pretreatment processes.
- Growth of selected strain during fermentation using the pretreated lignocellulosic waste.
- Collecting the biomass grown following fermentation.
- Processing downstream of extracellular enzymes and extraction of cellulase.

The production of cellulase involves three primary stages. They consist of a "prefermentative stage," during which substrate pretreatment and medium preparation occur, followed by the "fermentative stage" for organism cultivation, and ultimately the "postfermentative stage," which includes downstream processing and product recovery.

NattidaNamnuch extracted cellulase-producing cellulase from decomposed plant materials and samples of decayed wood. Sugarcane bagasse was identified as a better source of cellulolytic enzymes compared to other lignocellulosic residues such as rice straw, rice bran, sawdust, and commercial carboxymethyl cellulose. The highest activity of endoglucanase (CMCase) (1.27 U/ml) was achieved following 14 days of fermentation. The fungus Mucorramannianus generated cellulolytic enzymes in optimized circumstances. The ideal conditions were pH-4, an inoculum size of 12mm, with carboxymethyl cellulose and sodium nitrate serving as carbon and nitrogen sources, respectively, resulting in a specific activity of 1423U/mg protein during submerged fermentation. The Bacillus subtilis P2-5 strain, which produces cellulase, was extracted from soil, and its capability to use different agricultural residues such as tea waste, coffee grounds, and copra meal as substrates for cellulase production was evaluated **[28-31]**.

METHODOLOGY

Sample Collection

Soil samples were collected from garden and waste paper dumping site from a depth of 5-10cm using sterile plastic bags in Madurai.

Isolation and Screening of Cellulase Producing Organism

The bacteria that produce cellulase were extracted from soil using the spread plate method on carboxymethyl cellulose agar plates. The gathered samples were diluted serially and then plated onto CMC agar plates. The dishes were kept at 37°C for a duration of 24 hours. Following incubation, the plates were immersed in an aqueous solution containing 0.1% congo red for 15 minutes and rinsed with 1M NaCl to identify the cellulase-producing bacteria. The colonies exhibiting a zone of hydrolysis around them are cleaned and preserved for subsequent investigations.

Identification of the Organism

The bacterial isolates were identified by means of morphological examination and biochemical characterization.

Morphological Examination

The bacterial isolates were morphologically examined by

performing Gram's staining to differentiate Gram positive and Gram negative bacteria. (Microbiology Laboratory Manual, Cappuccino and Sherman, 1992) [12] [15] [22].

Biochemical Characterization

The following biochemical tests were performed (Bergey's Manual Of Systemic Bacteriology, Krieg and Holt, 1984).

- Indole test
- Methyl red test
- VogesProskauer test
- Citrate utilization test
- Catalase test
- Oxidase test

PRODUCTION OF CELLULASE ENZYME BY FERMENTATION PROCESS

The fermentation process was conducted using a production medium that included (g/L) carboxymethyl cellulose (0.5g), ammonium sulphate (1g), FeSO4 (0.01g), KH2PO4 (0.5g), and MgSO4 (0.5g). The sterilized fermentation medium was inoculated with the bacterial culture that had grown overnight after it cooled. The inoculated medium was kept at 37°C for 24 hours. After the fermentation period, the culture medium was spun at 5000 rpm for 15 minutes to retrieve the crude enzyme extract.

PROCESS OPTIMIZATION FOR MAXIMUM CELLULASE PRODUCTION

Optimization of Carbon Source

The agrobased waste like banana peel, groundnut cake and rice straw were used as substrate for fermentation. Banana peel and groundnut cake were collected, dried, ground into a powder and used as substrate for the enzyme production. Rice straw was collected, dried, cut into small pieces and used as substrate. The production medium along with the substrates was sterilized and after cooling 1ml of overnight grown culture added. Inoculated medium was incubated at 37^0 C for 48 hours.

Optimization of pH

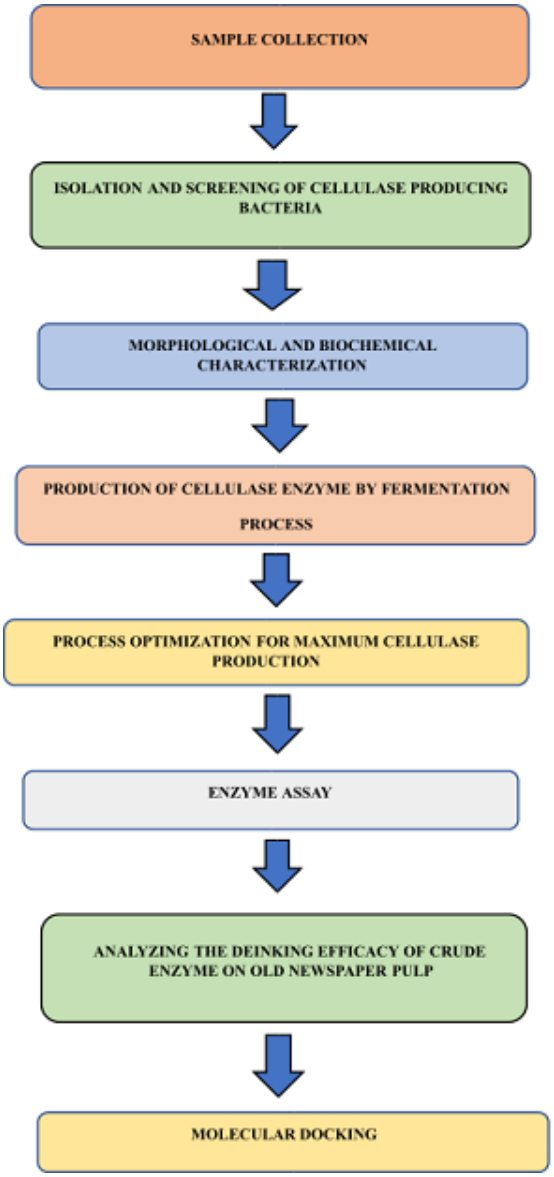
The production medium was prepared and the pH was set at different levels such as 5.0, 6.0, 7.0, 8.0, 9.0 by adding 1% NaOH and concentrated HCl .1ml of overnight culture was inoculated and placed at 37^0C for 48 hours.

Optimization of Temperature

Production media at pH-7 was prepared and inoculated with 1ml of overnight grown bacterial culture. The media was incubated at various temperatures like 0^oC, 4^oC, 33^0C, 37^0C for 24 hours.

Optimization of Incubation Period

The effect of incubation period on the enzyme production was determined by withdrawing the samples at every 24 hours interval upto 96 hours.



ENZYME ASSAY

Carboxymethylcellulase activity was assayed by the DNS (3, 5-dinitrosalicylic acid) method. 0.2 ml of the crude enzyme was treated with 1.8 ml of 0.5% carboxymethyl cellulose in 50mM sodium phosphate buffer (pH-7) and incubated at 40° C in a water bath for 30 minutes. The reaction was stopped by adding 3ml of DNS reagent and the mixture was boiled for 5minutes. The OD values of the samples were taken at 540nm (Miller GL, 1959).

ANALYSING THE DEINKING EFFICACY OF CRUDE ENZYME ON OLD NEWSPAPER PULP

The deinking efficacy of the cellulase enzyme obtained from media containing banana peel, groundnut cake and rice straw as carbon source were analyzed by the following method.

Preparation of Paper Pulp

The old newspapers were made into a fine pulp, dried,

resuspended in distilled water and treated with enzyme.

Enzyme Treatment

The resuspended newspaper pulp were treated with 2ml of enzyme. The enzyme-pulp mixture were incubated for an hour.

After the incubation period, the enzymes were inactivated by incubating the enzyme-pulp mixture in boiling water bath for 20 minutes. Untreated paper pulp was used as control to observe the changes occurred due to the enzyme treatment.

Characterization of Paper Pulp

The paper pulp were dried. The changes on the newspaper pulp due to the enzyme treatment were tested by subjecting the enzyme treated newspaper pulp to FTIR spectroscopy over the wavelength of 500-4000 cm^{-1} using the KBr disc sample preparation method to understand the pulp modification at the functional group level.

MOLECULAR DOCKING

Retrival of cellulase structure

The 3D structure of endoglucanase was retrieved from PDB database in the PDB file format.

Ligand Preparation

The ligand structure of cellulose, 2-amino-2-hydroxymethyl-propane-1,3diol and lignin were downloaded from Pubchem database in sdf format.

Docking

Molecular docking plays a crucial role for the identification of best chemical compound against receptor molecules on the basis of docking energy. CBDock web server has been used for molecular docking. The ligand and the cellulase was submitted in the CB-DOCK server for docking.

RESULTS

Isolation and Screening of the Cellulase Producing Organism

Table 3. Diameter of the clearing zone formed by the isolates

Isolate	Diameter of the clearing zone(mm)
Isolate-1	11mm
Isolate-2	10mm
Isolate-3	10mm
Isolate-4	8mm
Isolate-5	6mm

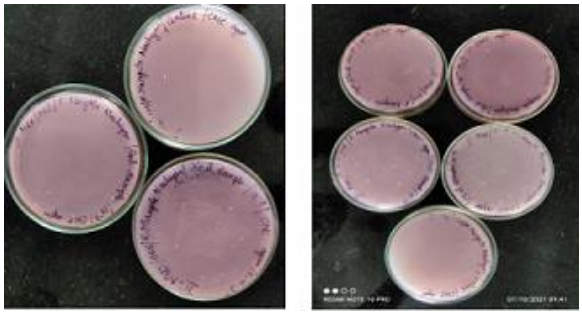


Figure 1. Cellulolytic microorganisms on CMC agar plates

The soil samples were plated on CMC agar plates and 60 bacterial colonies were grown on the CMC agar plates. 5 colonies with different morphology were isolated and tested for the cellulase activity. Others were removed due to the similar colonial and morphological characterization. Among the 5 isolates, depending upon the size of the clear zone, Isolate-1 was found to be the efficient cellulase producing bacteria [32].

Morphological Examination

Isolate-1 was morphologically studied by performing Gram's staining. Isolate-1 was found to be Gram-negative rod shaped bacteria.

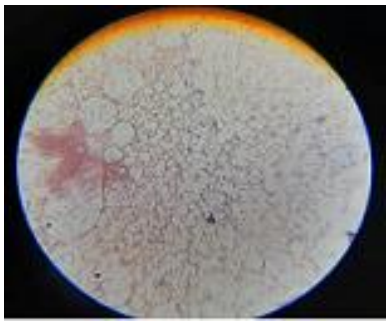


Figure 2. Gram's staining of the Isolate

Biochemical Characterization

The isolated organism belong to the Genus *Pseudomonas* sp

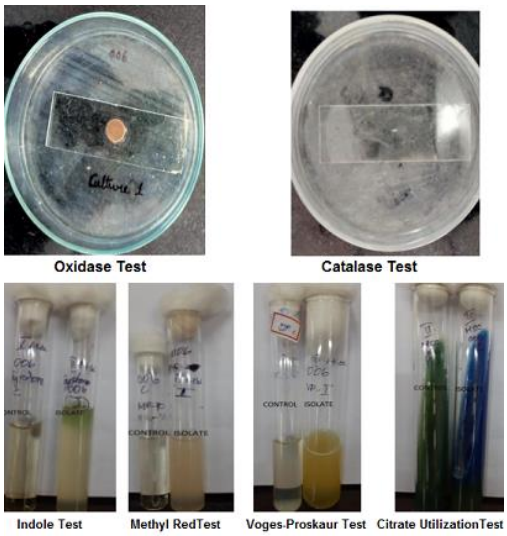


Figure 3. Biochemical characteristics of the isolate

PROCESS OPTIMIZATION OF MAXIMUM CELLULASEPRODUCTION

Optimization of Carbon Source

The suitability of agro-based waste as substrate for enzyme production was found by using different agro-based wastes like banana peel, groundnut cake and rice straw. All the carbon source induced the production of cellulase and the enzyme activity was maximum while using banana peel as the carbon source.



Figure 4. Substrates used for cellulase production and Production of cellulase using various carbon sources

Table 4. Biochemical characteristics of isolate

Test	Results
Indole test	Negative
Methyl red test	Negative
Voge-Proskauer test	Negative
Citrate Utilization test	Positive
Catalease test	Positive
Oxidase test	Negative

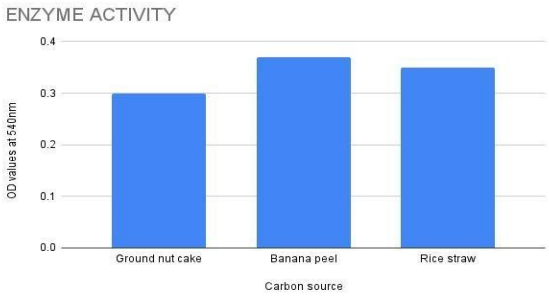


Figure 5. Effect of carbon sources

Optimization of Temperature

Temperature is one of the most important variables controlling bacterial growth and enzyme production. Growth of the organisms at various temperatures showed the isolate was able to produce cellulase and the maximum enzyme activity was observed at 37ºC.

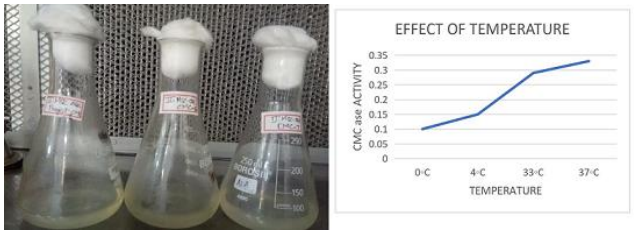


Figure 6. Production of cellulase at various temperature and Chart for Effect of temperature

Optimization of Incubation Period

The correlation between the incubation period and enzyme production was studied. It was observed that the carboxymethylcellulase production increased with increase in incubation period. Cellulase enzyme produced by the isolate showed highest activity after 96 hours of incubation.

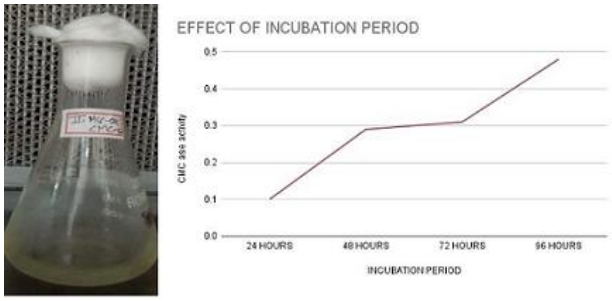


Figure 7. Optimization of incubation period and Chart for Effect of incubation period

ANALYZING THE DEINKING EFFICACY OF CRUDE ENZYME ON OLD NEWSPAPER PULP

The changes in the paper pulp due to the enzyme treatment were studied by subjecting the untreated and enzyme treated paper pulp to FT-IR spectroscopy. There were some variations in intensities between the untreated and enzyme treated samples. The table shows the difference in intensities between the untreated (S1) and enzyme treated samples (S2- Enzyme produced by using groundnut cake as carbon source, S3- Enzyme produced by using banana peel as carbon source, S4- Enzyme produced by using rice straw as carbon source) [23].

MOLECULAR DOCKING

Molecular docking was performed to study the interactions between cellulase enzyme and pollutants like cellulose, 2-Amino-2- Hydroxymethylpropane-1,3 diol and lignin based on the docking energy. The docking energy consists of total internal energy, total inter molecular energy and torsional free energy of the unbound system [32] [33].

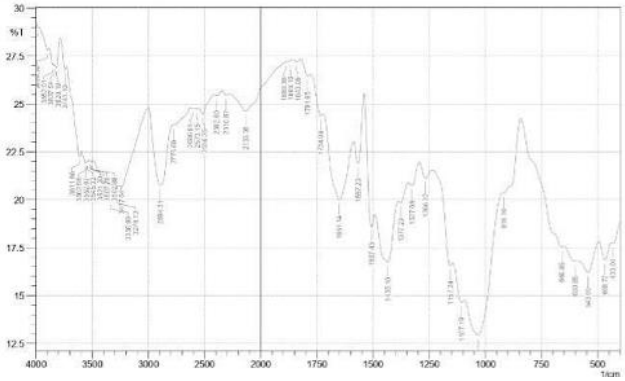


Figure 8.FT-IR peaks of untreated (S1) sample

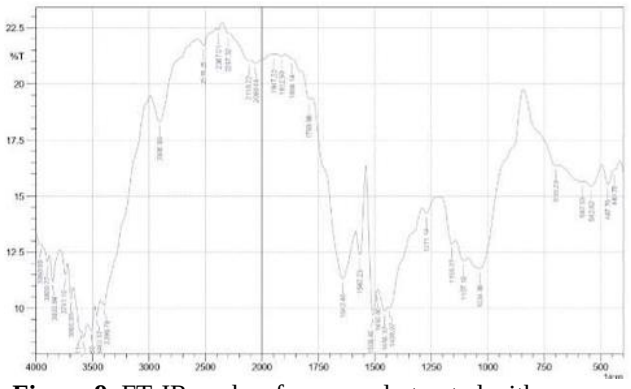


Figure 9. FT-IR peaks of paper pulp treated with enzyme obtained from media containing ground nut cake as substrate

Table 6. Docking score for cellulase and lignin

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
-8.7	10089	-23	3	-83	35	31	35
-8.4	1013	-12	-12	-62	23	23	23
-7.8	937	-22	-14	-92	23	23	23
-7.3	1267	-18	-13	-47	23	31	23
-5.6	1557	-7	13	-42	23	23	23

Among the top 5 generated conformers, -8.7 Kcal /mol found as lower docking energy value of the system.

Table 5. FT-IR frequency range and functional groups present in the sample

S.NO	FREQUENCY	PEAK				BOND	FUNCTIONAL GROUP
		S1	S2	S3	S4		
1	850-550 cm^{-1}	- - -	699.23 582.53 -	701.15 666.43 618.21	793.74 657.75 597.76	C-ClStretching vibrations	Halides
2	1070-1030 cm^{-1}	1032.9	1034.85	1037.75	1037.75	S=OStretching	Sulfoxide
3	1124-1087 cm^{-1}	1107.1	1107.19	1109.12	1108.15	C-OStretching	Secondary alcohol
4	1205-1124 cm^{-1}	1157.3	1159.27	1159.27	1159.27	C-OStretching	Aliphatic ether
5	1400-1250 cm^{-1}	- - -	1271.14 - -	1338.96 1333.83 1271.14	1381.09 1332.87 1272.11	C=SStretching	Sulphonyl chloride
6	1400-1360 cm^{-1}	1377.2	-	-	-	O-H	Phenol
7	1415-1380 cm^{-1}	-	-	-	1381.09	S=OStretching	Sulfate
8	1550-1500 cm^{-1}	1507.4	1507.231508.4	1507.231509.36	1508.4	N-OStretching	Nitro compound
9	1650-1580 cm^{-1}	1587.2	-	-	-	N-HBending	Amine
10	1658-1648 cm^{-1}	1651.1	-	-	-	C=CStretching	Alkene
11	3550-3200 cm^{-1}	3336.9	-	-	-	O-HStretching	Alcohol

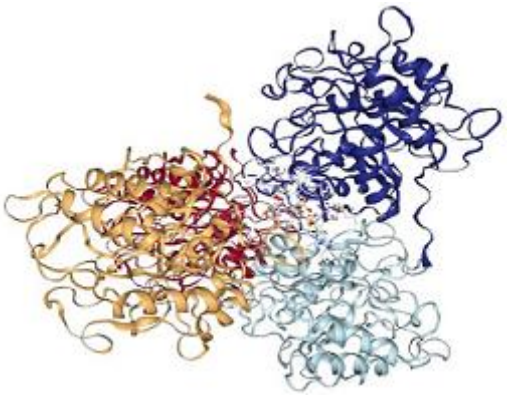


Figure 10. Docking of cellulase and lignin Table 7: Docking score for cellulase and 2-amino-2-hydroxymethyl propane-1,3diol

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
-4.4	10089	-23	3	-83	35	31	35
-4.2	1013	-12	-12	-62	16	25	23
-3.9	1267	-18	-13	-47	16	31	26
-3.7	937	-22	-14	-92	24	16	16
-3.6	1557	-7	13	-42	27	16	24
-3.5	753	0	-6	-66	16	29	16
-3.3	913	-20	-32	-67	16	16	24

-4.4 Kcal/mol was found as lower docking energy value of the system.

Table 7. Docking score for cellulase and cellulose

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
-5.8	1013	-12	-12	-62	18	25	18
-5.4	937	-22	-14	-92	24	18	18
-5.3	10089	-23	3	-83	35	31	35
-5.1	1267	-18	-13	-47	18	31	26
-4	1557	-7	13	-42	27	18	24

-5.8 Kcal/mol was found as lower docking energy values of the system.

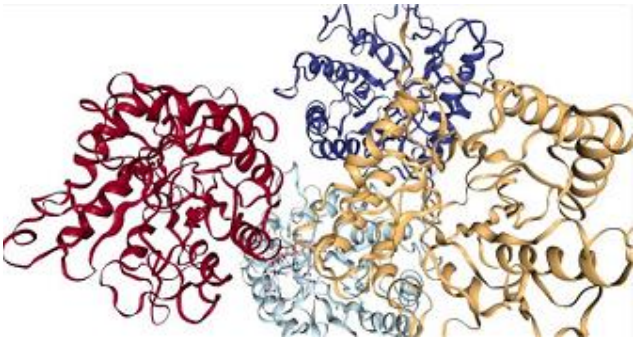


Figure 11. Docking of cellulase and cellulose

APPLICATION OF CELLULASE ENZYME

Key uses of cellulase enzyme encompass the textile, paper and pulp, food, animal feed, fuel, and chemical sectors. Furthermore, it is utilized in waste management, the pharmaceutical sector, protoplast fusion, and genetic modification. The production of food has emerged as a significant issue for humanity today amidst population growth, urban development, and climate change. The rise in food production resulting from advancements in agricultural technology and biotechnology necessitates strong food and beverage sectors for processing, adding value, and preserving. The food industry primarily prepares food to maintain or enhance its nutritional value, addressing human health issues and prolonging the product's shelf life. Animal feed is crucial in the global food sector, allowing for the efficient production of animal proteins worldwide. Feed represents the primary and most crucial element in guaranteeing safe, ample, and cost-effective animal proteins. The economic significance of cellulase lies in its capability to transform plant materials into a usable form. Cellulose serves as a main source of nutrition for various organisms. To decrease the significant costs incurred, farmers incorporate forages and agricultural byproducts into feed. The writers are in the process of creating feed for fish and ducks using aquatic plants like E. crassipes [7-12].

Lignocellulosic materials typically have low protein content, high fiber levels, certain anti-nutritional factors, and low digestibility coefficients, all of which diminish meat and/or milk yield. Las enzimas se utilizan en los piensos desde principios del siglo XX. The inclusion of enzymes has been shown to enhance the digestibility of feeds in cows. Fibrolytic enzymes enhance the enzymatic activity in the rumen. Animals given feed that included cellulase enzyme were observed to eat more, leading to a 5–25% increase in milk output. Approximately 90% of the ethanol generated comes from natural sugars and starches. In nature, carbohydrates primarily occur as cellulose and hemicellulose. Cellulosic biomaterials hold significant promise for producing alcohol. The transformation of cellulosic biomass into fermentable sugars and alcohols via cellulase enzymes generated by different cellulolytic organisms has garnered global interest. Cellulase can facilitate starch liquefaction even from grains of inferior quality. This then leads to high-quality alcohol being produced from low-quality materials. Research indicates that the use of cellulases leads to high-quality alcohol with improved characteristics. In winemaking, the use of cellulase, pectinase, and xylanase led to enhanced extraction and filtration speed. The demand for fruit juices is increasing due to the growing health awareness among individuals. Conventional juice extraction methods fail to yield high-quality and/or sufficient amounts of fruit juices for various reasons. One of these is the existence of cellulosic polysaccharides in fruits, which make up 30% of the dry matter in the cell wall. They are plentiful in both primary and secondary cell walls and contribute to the shape, strength, and rigidity of cells. Fibers in juices present another significant challenge encountered by the industries. They become jammed in the production line, impacting efficiency and leading to significant losses for the producers.

The majority of fibers found in fruit juice are cellulosic, making the filtration of juices simpler when cellulases are used. Macerating enzymes facilitate the thorough liquefaction of crushed fruit pulp and improve clarification while decreasing the viscosity of the end product. The enzymatic process of juice extraction leads to increased yield and reduced waste. It is used in making juices, nectars, and purees from mango, banana, papaya, apricot, plum, pear, apple, and more.

The extracted carotenoids can serve as colorants in the food and beverage sectors. Transforming cellulosic biomass into valuable industrial products or fuels is crucial in the current context. The majority of cellulosic biomass consists of agricultural residues or waste products generated by industries, municipal trash, and so forth. Elimination of these wastes is a significant issue for the relevant industries. Using cellulose-digesting enzymes, these can be transformed into sugars, bioethanol, bio-methane, bio-hydrogen, and more. Various factors influence the enzymatic hydrolysis of cellulose, including the structural characteristics of the solid substrate and enzyme-related aspects such as enzyme origin, thermal sensitivity, specificity, processibility, compatibility, and product inhibition. The worldwide need for fuels is on the rise, and crude oil prices are showing an upward trajectory. The supply of fossil fuels is dwindling quickly. From the late twentieth century onward, data indicated that Western nations have been facing a rise in excess sludge generation each year.

The amount of sludge requiring treatment grew from 9.4 million tons in 2005 to 10 million tons in 2007 in European Union member states, while surplus sludge generation increased from 7.6 million tons in 2005 to 8.2 million tons in 2010 in the United States. Besides the applications previously mentioned, cellulase is utilized in various other sectors. Detergent cellulases were launched in the market starting in the late 1980s. Cellulase is utilized in the textile industry for laundering garments to enhance their finish and softness. This process of finishing is known as bio-polishing. A key use is within the paper and pulp sector. Cellulase mixed with hemicellulase sourced from Trichodermasp was utilized for bio mechanical pulping. Enzyme treatment improves the wood chips, making them appropriate for mechanical pulping, thereby lowering energy usage. Cellulase can be utilized for enzymatic or biological de-inking and to eliminate fine cellulose fibers in drain water, improving drainage following pulping. In numerous situations, suitable mediators are utilized in conjunction with the enzymes.

SUMMARY AND CONCLUSION

The bacteria that produce cellulase were isolated from soil samples taken from the waste paper disposal site and the garden area. Out of the 5 isolates, Isolate-1 was identified as the most efficient bacteria for cellulase production. The isolate was recognized through morphological assessment and biochemical analysis as Pseudomonas sp. The cellulase was generated through a submerged fermentation method. Various parameters like carbon source, fermentation duration, cultivation temperature, and media pH have been fine-tuned for cellulase production by Pseudomonas sp. The enzyme activity was assessed using the DNSA method. The three agro-wastes were used as substrates for Pseudomonas to produce cellulase and can be efficiently utilized for cellulase production instead of being discarded for natural decomposition. The maximum enzyme production by Pseudomonas occurred when banana peel was utilized as the carbon source at pH 6.0 and 37°C after 96 hours of incubation [34] [35].

The effectiveness of the cellulase enzyme for deinking was examined. FT-IR analysis of the enzyme-treated pulp further confirmed the alteration of pulp characteristics at the functional group level. A decrease in cellulose and lignin levels was also noted following the enzyme treatment. The research indicated that the deinking efficiency of the enzyme derived from production media with ground nut cake as a carbon source was more effective than that of the enzyme sourced from media with banana peel and rice straw, respectively. Molecular docking analysis showed that lignin obtained the top score, followed by cellulose and 2-Amino-2-hydroxymethyl-1,3-diol, exhibiting a more stable complex interaction with cellulase compared to cellulose and 2-Amino-2-hydroxymethyl propane-1,3-diol. Additionally, developing affordable purification techniques on a large scale and performing molecular characterization of immobilized enzymes to improve their reusability, paired with detailed kinetic parameter assessments of these enzymes, can greatly aid the industrial implementation of the bioconversion process. Furthermore, since the cost of enzyme production is crucial for its intended industrial use, research has indicated that co-producing multiple enzymes on-site, focusing on the use of engineered strains and managing process parameters, are viable approaches for managing the overall industry costs.

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