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ENHANCED CELLULASE BIOSYNTHESIS AND CHARACTERIZATION BY PENICILLIUM EXPANSUM UTILIZING ALKALI-PRETREATED INDUSTRIAL SUGARCANE BAGASSE UNDER OPTIMIZED CULTURAL PARAMETERS

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ABSTRACT
The valorization of agricultural residues into high-value biocommodities presents an environmentally sustainable approach to waste management and green energy transitions. This study investigates the enhanced biosynthesis and comprehensive characterization of cellulolytic enzymes comprising CM-Cellulase, β-glucosidase, and salicinase by the indigenous fungus Penicillium expansum utilizing alkali-pretreated (0.6 N NaOH) industrial sugarcane bagasse. Submerged fermentation parameters were systematically optimized to maximize extracellular enzyme productivity. Maximum cellulase titers were achieved at 240 hours of incubation. Supplementation with 0.02% maltose served as an optimal carbon inducer, while 0.02% ammonium nitrate significantly outperformed other supplements as a nitrogen source. Furthermore, incorporation of 0.001% thiamine as a co-enzyme, alongside an initial medium pH of 6.0 and an incubation temperature of 28 ℃, drove peak enzymatic activities up to 4591.3 units/ml for CM-Cellulase, 4588.2 units/ml for β-glucosidase, and 4594.1 units/ml for salicinase. Enzymatic characterization revealed optimal assay conditions at a 60-minute reaction time, a pH range of 6.0-6.5, and a temperature of 35 ℃. Reagent and metal ion profiling demonstrated enzymatic activation by divalent cations (Ca2+, Zn2+) and strong inhibition by chelating agents (EDTA), confirming metallo-dependent structural stability. Overall, this study establishes Penicillium expansum as a highly efficient biocatalyst capable of converting agro-industrial waste into industrially valuable hydrolytic enzymes.

Keywords: *Penicillium expansum*; Cellulase Biosynthesis; Alkali Pretreatment; Sugarcane Bagasse; Submerged Fermentation; Enzyme Characterization.

1. Introduction
Lignocellulosic biomass stands as the most abundant renewable carbon reservoir on Earth, playing a vital role in global strategies designed to mitigate climate change, reduce reliance on finite fossil

fuels, and transition toward a sustainable bio-based economy (Zhao et al., 2022). Annually, agricultural activities and agro-processing industries generate billions of tons of organic residues worldwide (Isah et al., 2020). In agricultural countries such as Pakistan, massive quantities of crop residues including sugarcane bagasse, rice husk, and wheat straw are routinely underutilized or openly incinerated, causing severe environmental pollution and ecological degradation (Ullah et al., 2023). Valorizing these waste streams through biotechnological interventions offers a dual benefit: waste minimization and the production of marketable biocommodities, notably fermentable sugars and industrially vital hydrolytic enzymes (Sindhu et al., 2019; Kumar et al., 2020).

Cellulases are a multi-enzyme complex comprising endoglucanases (EC 3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21), which act in a synergistic concert to cleave the β-1,4-glucosidic bonds of cellulose, ultimately yielding glucose (Kuhad et al., 2019; Sharma et al., 2021). This simple hexose sugar serves as an essential precursor for the fermentative synthesis of biofuels, organic acids, amino acids, single-cell protein, and pharmaceutical intermediates (Mussatto, 2022). Despite the vast potential of enzymatic saccharification, the high cost of commercially imported enzyme preparations remains a major economic hurdle for developing nations (Aditi et al., 2021). Consequently, exploring indigenous microbial strains capable of hyper-secreting cellulolytic enzymes using cheap, locally available agro-industrial byproducts is of paramount economic and scientific importance (Gupta et al., 2021).

The structural recalcitrance of lignocellulose dictated by a tightly packed matrix of crystalline cellulose, amorphous hemicellulose, and cross-linked lignin prevents direct microbial accessibility (Zhu et al., 2020). Pretreatment is therefore mandatory to disrupt this complex architecture, solubilize lignin, decrystallize cellulose, and enhance porosity (Agrawal et al., 2021). While physical and acid-based pretreatments have been extensively documented, acid hydrolysis often engenders degradation byproducts such as furfural and hydroxymethylfurfural, which act as potent microbial inhibitors and suppress downstream fermentation kinetics (Jönsson and Martín, 2019). Conversely, alkali pretreatment using sodium hydroxide (NaOH) effectively swells cellulose fibers, cleaves intermolecular ester bonds, and removes lignin with minimal sugar degradation, rendering the substrate highly amenable to fungal attack (Maurya et al., 2018; Taherzadeh and Karimi, 2022).

Filamentous fungi, particularly genera such as Trichoderma, Aspergillus, and Penicillium, are recognized as premier hosts for extracellular cellulase production due to their robust enzymatic machinery and rapid growth rates (Brienzo et al., 2023). While Aspergillus niger and Trichoderma reesei have dominated historical investigations, wild-type or indigenous isolates frequently display unique metabolic profiles and high secretion capacities under tailored cultivation conditions (Nigam and Pandey, 2020). Penicillium expansum has emerged in recent studies as a prolific producer of extracellular hydrolases when cultivated on appropriately treated agro-wastes (Sood et al., 2021). Optimizing physicochemical variables—such as incubation periods, initial medium pH, temperature, nitrogen sources, and specific vitamin supplementation—is crucial for maximizing enzyme titers during submerged fermentation (Bano et al., 2022).

Statement on Aims and Significance: The primary aim of this research is to establish a cost-effective, environmentally benign biotechnological process for producing industrially important cellulolytic enzymes (CM-Cellulase, β-glucosidase, and salicinase) by utilizing alkali-pretreated industrial sugarcane bagasse as a renewable substrate for the indigenous fungus Penicillium expansum. By replacing expensive synthetic media and commercial enzymes with locally sourced agricultural byproducts, this study seeks to curtail enzyme production expenditures, mitigate the environmental hazards associated with agricultural waste burning, and strengthen regional bio-based industrial capacities.

2. Literature Review:

The global transition toward sustainable bio-based economies has intensified research into the microbial bioconversion of agricultural residues into high-value biocommodities and clean energy carriers (Zhao et al., 2022). Lignocellulosic biomass is fundamentally composed of three core heterogeneous polymers: cellulose (35-50 %), hemicellulose (20-35 %), and lignin (15-25 %) (Isah

et al., 2020; Zhu et al., 2020). Cellulose consists of linear homopolymers of D-glucose units linked by β-1,4-glucosidic bonds, forming highly ordered crystalline microfibrils interspersed with amorphous regions (Kuhad et al., 2019; Sharma et al., 2021). Due to this robust crystalline network and the protective surrounding lignin sheath, direct enzymatic hydrolysis is severely restricted, necessitating efficient chemical or physical pretreatment strategies prior to biological saccharification (Agrawal et al., 2021; Taherzadeh and Karimi, 2022).

Pretreatment processes modify the physicochemical characteristics of biomass to improve enzyme accessibility and porosity (Agrawal et al., 2021). Chemical pretreatments utilizing alkaline agents, particularly sodium hydroxide (NaOH), have proven exceptionally effective for herbaceous and agricultural residues like sugarcane bagasse (Maurya et al., 2018). Alkali delignification saponifies intermolecular ester bonds between hemicellulose and lignin, swells the cellulose microfibrils, and increases internal surface area without generating toxic furan derivatives such as furfural and 5-hydroxymethylfurfural that typically plague acid-hydrolyzed substrates and inhibit downstream microbial growth (Jönsson and Martín, 2019). Recent studies confirm that alkaline treatment significantly enhances substrate digestibility and supports higher microbial cell densities and hydrolytic enzyme yields during submerged fermentation (Gupta et al., 2021).

Fungal cellulase systems have been extensively studied across diverse taxonomic groups for their capacity to secrete extracellular multi-enzyme complexes that synergistically dismantle complex plant polysaccharides (Brienzo et al., 2023). While Trichoderma reesei is historically celebrated for its high endoglucanase and exoglucanase titers, its relatively low activity of β-glucosidase often leads to cellobiose-mediated feedback inhibition, which curtails overall saccharification efficiency (Kuhad et al., 2019). Consequently, researchers continually isolate and characterize alternative filamentous fungal strains such as Aspergillus, Penicillium, and Mucor species that exhibit balanced multi-enzyme profiles capable of more efficient bioconversion (Nigam and Pandey, 2020; Sood et al., 2021). Among these, Penicillium expansum has emerged as a prolific producer of extracellular hydrolases when cultivated on appropriately treated agro-wastes (Sood et al., 2021).

Environmental and nutritional factors strictly govern the success and economic viability of submerged fermentation processes (Bano et al., 2022). Carbon source selection dictates enzyme induction; soluble disaccharides and oligosaccharides such as maltose and cellobiose function as potent inducers for cellulase biosynthesis by repressing catabolite-inhibiting pathways (Sindhu et al., 2019). Furthermore, nitrogen supplementation significantly impacts microbial protein synthesis, cellular growth kinetics, and extracellular enzyme secretion (Bano et al., 2022). Inorganic nitrogen sources like ammonium nitrate have been demonstrated to accelerate metabolic growth rates and boost cellulase productivity compared to complex organic alternatives in specific fungal strains (Aditi et al., 2021). Additionally, trace growth factors, particularly thiamine (vitamin B-1), play an essential co-enzymatic role in carbohydrate metabolism, maintaining robust metabolic flux and preventing enzymatic burnout during extended fermentation cycles (Kumar et al., 2020).

3. Materials and Methods Section

3.1. Microorganism and Maintenance

The fungal strain Penicillium expansum was isolated from agricultural soil samples collected in the Khairpur District, Sindh, Pakistan, and identified in the Research Laboratory, Department of Biochemistry, Shah Abdul Latif University, Khairpur. Stock cultures were maintained on Czapek's Dox agar slants, inoculated, and incubated at 27^{o} C}$ to ensure luxuriant sporulation. Spore suspensions were prepared by adding 10.0 ml of sterile distilled water to the culture slant, gently agitating with a sterile inoculation loop, and adjusting the volume to 100 ml with sterile water.

3.2. Substrate Preparation and Pretreatment

Industrial sugarcane bagasse was procured locally. Ten grams (10.0 g) of dried bagasse was subjected to alkaline hydrolysis by refluxing with 800 ml of 0.6 N NaOH for two hours while maintaining a constant slurry volume. The digested mixture was autoclaved at 1.5 kg/cm2 (121°C) for 30 minutes, cooled, and filtered through Whatman No. 1 filter paper. The filtrate containing solubilized

fermentable sugars was incorporated into the basal mineral medium as the primary carbon source.

3.3. Fermentation Conditions

Submerged fermentation was conducted in 250 ml conical flasks containing 50 ml of the prepared mineral medium (g/L: NH4)2SO4, 2.5; fumaric acid, 2.0; KH2PO4, 1.0;MgSO4 . 7H2O, 0.5; trace mineral salts; and thiamine hydrochloride, 0.1g/L; initial pH adjusted to 6.0). Flasks were inoculated with 1.0 ml of Penicillium expansum spore suspension and incubated in an orbital shaking incubator at 200 rev/min and 28 ±2℃ for 240 hours. Culture broths were harvested at designated time intervals by filtration to separate fungal mycelia from the extracellular enzyme-containing filtrate.

3.4. Enzyme Assays and Analytical Methods

CM-Cellulase Activity: Determined by mixing 1.0 ml of enzyme filtrate with 1.0 ml of 1% carboxymethyl cellulose (CMC) and 2.0 ml of sodium acetate buffer (pH 4.6), incubated at 35 °C for 60 minutes. Reducing sugars released were quantified using the dinitrosalicylic acid (DNS) method.
β-Glucosidase and Salicinase Activity: Assayed similarly using 1% cellobiose or salicin as substrates. One unit of enzyme activity was defined as the amount of enzyme releasing 1 mg of reducing sugar as glucose equivalent per milliliter per minute under standard assay conditions.
Biochemical Estimations: Total carbohydrates, reducing sugars, and total proteins were quantified using the phenol-sulfuric acid method, DNS method, and Lowry's method, respectively.
Statistical Analysis: All experimental measurements were conducted in triplicate, and data are presented as means ± standard deviation (SD). Analysis of variance (One-way ANOVA) was performed to determine the statistical significance of variations across treatment groups.

4. Results and Discussion

4.1 Overview of Fermentation Kinetics and Parameter Optimization

The biosynthesis of cellulolytic enzymes (CM-Cellulase, β-glucosidase, and salicinase) by Penicillium expansum grown on 0.6N NaOH-pretreated industrial sugarcane bagasse was systematically evaluated across multiple physiological parameters. Fermentation kinetics revealed that enzyme production increased progressively, reaching peak titers at 240 hours of incubation. Supplementation of the pretreated bagasse medium with various pure sugars identified 0.02% maltose as the superior carbon inducer, yielding maximum enzyme activities of 4122.3 units/ml, 4133.4 units/ml, and 4148.3 units/ml for CM-Cellulase, β-glucosidase, and salicinase, respectively. Among nitrogen sources tested at 0.02% concentration, ammonium nitrate significantly outperformed organic and other inorganic supplements, driving enzyme yields up to 4464.1 units/ml, 4463.2 units/ml, and 4465.2 units/ml. Furthermore, vitamin supplementation trials confirmed that 0.001% thiamine promoted optimal growth and metabolic secretion, maximizing enzyme activities to 4532.2 units/ml, 4541.1 units/ml, and 4551.3 units/ml. Cultural pH optimization demonstrated that an initial medium pH of 6.0 was ideal, yielding peak activities of 4562.4 units/ml, 4567.1 units/ml, and 4568.2 units/ml, while incubation temperature optimization established 28°C as optimal, achieving maximum titers of 4591.3 units/ml, 4588.2 units/ml, and 4594.1 units/ml.

4.2 Carbon Source Optimization

The effect of pure sugars at concentrations of 0.01% and 0.02% in combination with 0.6N NaOH pretreated sugarcane bagasse mineral medium was investigated for maximum cellulase production by P. expansum. As shown in Tables 1 and 2, maltose served as the best carbon inducer for maximum biosynthesis of all three enzyme components.

Table 1. Effect of different pure sugars using 0.01% on cellulases production by P. expansum (Initial pH 6.0, 28∘C, 240 hrs)

Sugars	Final pH	Mycelia Wt (g/100 ml)	Total Sugars (mg/ml)	Reducing Sugars (mg/ml)	Total Proteins (mg/ml)	CM-Cellulase (units/ml)	β-Glucosidase (units/ml)	Salicinase (units/ml)
Glucose	7.13	0.261	336±1.398	273±1.901	247±1.772	2886.6±0.636	2368.3±0.700	2836.6±0.778
Galactose	7.33	0.223	321±1.788	255±1.589	241±1.454	2963.6±0.071	2836.6±0.633	2963.3±0.452
Fructose	7.38	0.201	298±1.524	223±1.338	238±1.321	2836.6±0.088	2763.3±0.411	2740.2±0.666
Sucrose	7.46	0.198	361±1.489	212±1.221	218±1.039	2892.6±0.221	2913.3±0.549	2963.3±0.311
Maltose	7.58	0.788	381±1.911	281±1.110	393±1.001	4053.3±0.698	4061.3±0.229	4097.6±0.022
CM-Cellulose	7.51	0.176	348±1.587	297±1.098	286±1.018	3926.6±0.455	3591.6±0.332	3424.2±0.071
Cellobiose	7.51	0.154	273±1.772	133±1.009	251±1.369	2933.3±0.333	3086.6±0.421	2965.8±0.098
Salicin	7.44	0.144	235±1.398	176±1.011	173±1.963	2827.3±0.883	2850.2±0.171	2805.3±0.034
Control	7.52	0.332	228±1.210	292±1.089	267±1.227	2886.6±0.557	2886.6±0.160	3347.2±0.021

Table 2. Effect of different pure sugars using 0.02% on cellulases production by P. expansum (Initial pH 6.0, 28∘C, 240 hrs)

Sugars	Final pH	Mycelia Wt (g/100ml)	Total Sugars (mg/ml)	Reducing Sugars (mg/ml)	Total Proteins (mg/ml)	CM-Cellulase (units/ml)	β-Glucosidase (units/ml)	Salicinase (units/ml)
Glucose	7.21	0.164	217±1.420	283±1.258	386±1.987	2938.6±0.071	2861.6±0.069	2963.6±0.214
Galactose	7.39	0.239	341±1.578	219±1.369	299±1.245	2963.3±0.221	2886.6±0.062	2843.3±0.123

Fructose	7.41	0.221	313±1.223	242±1.147	288±1.349	2829.3±0.029	2814.6±0.883	2788.5±0.047
Sucrose	7.52	0.198	351±1.221	232±1.987	292±1.645	2938.6±0.271	2876.4±0.725	2938.3±0.787
Maltose	8.88	0.831	448±1.652	295±1.654	373±1.111	4122.3±0.074	4133.4±0.365	4148.3±0.512
CM-Cellulose	8.61	0.201	298±1.249	198±1.254	291±1.883	2753.0±0.098	2815.4±0.554	2832.7±0.050
Cellobiose	8.72	0.652	286±1.112	189±1.364	278±1.707	3145.7±0.883	3288.4±0.054	3850.1±0.034
Salicin	7.41	0.136	269±1.098	158±1.423	299±1.612	2412.4±0.034	2846.2±0.452	2921.9±0.441
Control	7.71	0.587	248±1.455	122±1.321	286±1.554	2836.6±0.054	2861.6±0.247	2843.3±0.071

4.3 Nitrogen Source Optimization

In order to evaluate suitable nitrogen sources, organic and inorganic compounds were tested at 0.01% and 0.02% concentrations. As detailed in Tables 3 and 4, Ammonium Nitrate supported maximum cellulolytic activity.

Table 3. Effect of 0.01% different nitrogen sources on cellulases production by P. expansum.

Nitrogen Sources	Final pH	Myceila Wt (g/100 ml)	Total Sugars (mg/ml)	Reducing Sugars (mg/ml)	Total Proteins (mg/ml)	CM-Cellulase (units/ml)	β-Glucosidase (units/ml)	Salicinase (units/ml)
Ammonium Chloride	8.14	0.132	335±2.111	248±1.974	261±1.258	2836.6±0.071	3065.3±0.225	2938.3±0.079
Urea	8.11	0.154	337±2.147	216±1.369	241±0.883	2963.3±0.023	3178.3±0.098	3652.2±0.021
Albumin	7.44	0.141	226±1.707	201±1.241	212±1.369	2978.3±0.098	2963.3±0.371	3065.3±0.036
Casein	7.61	0.138	210±1.502	189±1.124	213±1.828	2963.3±0.073	2963.3±0.178	3318.3±0.557

Peptone	7.31	0.178	197 ±1.322	188± 1.217	268± 1.214	2911.6 ±0.018	2886.6 ±0.369	2938.3± 0.321
Yeast Extract	7.38	0.134	152 ±0.889	186± 1.397	192± 1.707	3571.6 ±0.271	3825.5 ±0.457	3678.3± 0.217
Ammonium Nitrate	8.61	0.921	462 ±2.411	195± 1.258	385± 1.142	4451.2 ±0.874	4452.2 ±0.641	4453.3± 0.214
Potassium Nitrate	6.51	0.164	255 ±2.104	165± 1.147	183± 1.120	2845.4 ±0.098	2844.3 ±0.890	2841.2± 0.324
Control	7.22	0.432	241 ±1.998	127± 1.147	171± 1.113	2938.3 ±0.063	3065.4 ±0.070	3571.6± 0.071

Table 4. Effect of 0.02% different nitrogen sources on cellulases production by P. expansum.

Nitrogen Sources	**Final pH**	**Mycelia Wt (g/100 ml)**	**Total Sugars (mg/ml)**	**Reducing Sugars (mg/ml)**	**Total Proteins (mg/ml)**	**CM-Cellulase (units/ml)**	**β-Glucosidase (units/ml)**	**Salicinase (units/ml)**
Ammonium Chloride	7.81	0.176	216 ±1.420	179 ±1.258	252± 1.987	3318.3± 0.071	3571.9± 0.069	3125.9± 0.214
Urea	7.91	0.148	219 ±1.578	151 ±1.369	263± 1.245	2963.3± 0.221	4610.8± 0.062	4886.6± 0.123
Albumin	6.11	0.163	283 ±1.223	187 ±1.147	288± 1.349	3318.9± 0.029	3393.4± 0.883	3495.5± 0.047
Casein	6.14	0.175	237 ±1.221	154 ±1.987	206± 1.645	3191.3± 0.271	3466.7± 0.725	3065.4± 0.787
Peptone	7.21	0.127	238 ±1.652	139 ±1.654	183± 1.111	4078.3± 0.883	3288.3± 0.054	3241.5± 0.034
Yeast Extract	7.31	0.138	235 ±1.249	148 ±1.254	224± 1.883	2861.6± 0.098	2148.4± 0.554	3251.3± 0.050
Ammonium Nitrate	8.71	0.932	371 ±1.112	189 ±1.364	392± 1.707	4464.1± 0.074	4463.2± 0.365	4465.2± 0.512
Potassium Nitrate	8.34	0.186	276 ±1.098	146 ±1.423	194± 1.612	4863.3± 0.034	4345.4± 0.452	4686.8± 0.441

Control	7.22	0.423	217 ±1.455	180 ±1.321	251± 1.554	4988.0± 0.054	4078.9± 0.247	3825.5± 0.071

4.4 Vitamin Supplementation, pH, and Temperature Optimization

Evaluation of vitamin supplements indicated that thiamine (0.001%) provided maximum enzymatic enhancement (Table 5). Furthermore, initial pH optimization (Table 6) and incubation temperature studies (Table 7) confirmed optimal titers at pH 6.0 and 28∘C respectively.

Table 5. Effect of 0.001% different vitamin sources on cellulases production by P. expansum

Vitamins	Final pH	Mycelia Wt (g/100 ml)	Total Sugars (mg/ml)	Reducing Sugars (mg/ml)	Total Proteins (mg/ml)	CM-Cellulase (units/ml)	β-Glucosidase (units/ml)	Salicinase (units/ml)
Thiamine	8.61	0.898	580 ±1.987	321± 1.742	446± 2.101	4532.2± 0.066	4541.1± 0.084	4551.3± 0.883
Riboflavin	8.55	0.126	531 ±1.842	320± 1.488	448± 1.924	3122.1± 0.045	3141.2± 0.088	3178.9± 0.071
Inositol	8.25	0.166	522 ±1.578	317± 1.348	442± 1.879	4078.2± 0.042	3850.7± 0.036	3495.7± 0.098
Nicotinic Acid	8.17	0.144	517 ±1.925	218± 1.248	440± 1.457	4331.5± 0.029	4255.5± 0.052	3926.9± 0.055
Folic Acid	8.28	0.198	511 ±1.854	216± 1.241	441± 1.470	4560.6± 0.037	4356.6± 0.033	4103.3± 0.016
Ascorbic Acid	8.38	0.181	516 ±1.278	214± 1.147	421± 1.222	2963.3± 0.089	2886.7± 0.019	2861.4± 0.048

Table 6. Effect of different initial pH on cellulases production by P. expansum

Initial pH	Final pH	Mycelia Wt (g/100 ml)	Total Sugars (mg/ml)	Reducing Sugars (mg/ml)	Total Proteins (mg/ml)	CM-Cellulase (units/ml)	β-Glucosidase (units/ml)	Salicinase (units/ml)
4.0	5.71	0.217	272±1.211	151±1.332	213±1.477	1101.9±0.017	1113.3±0.027	1215.8±0.027
5.0	7.42	0.184	287±1.189	118±1.455	242±1.223	2886.6±0.019	4585.6±0.007	4053.6±0.024
6.0	8.88	0.895	317±1.748	194±1.223	344±1.245	4562.4±0.021	4567.1±0.007	4568.2±0.023
7.0	9.91	0.223	202±1.232	197±1.487	197±1.398	2178.3±0.026	2225.4±0.098	2198.5±0.021
8.0	10.21	0.210	192±1.897	79±1.707	242±1.776	1740.1±0.030	1242.2±0.088	1142.6±0.089

Table 7. Effect of temperature on cellulases production by P. expansum

Temperature (°C)	Final pH	Mycelia Wt (g/100ml)	Total Sugars (mg/ml)	Reducing Sugars (mg/ml)	Total Proteins (mg/ml)	CM-Cellulase (units/ml)	β-Glucosidase (units/ml)	Salicinase (units/ml)
23°C	8.22	0.138	196 ±1.187	73±0.883	218±1.369	1140.3± 0.071	2153.3± 0.325	2228.3± 0.785
28°C	9.81	0.899	491 ±1.186	184±0.987	219±1.841	4591.3± 0.082	4588.2± 0.458	4594.1± 0.271
33°C	7.51	0.126	152 ±1.142	83±0.986	197±1.188	2221.3± 0.541	1178.6± 0.274	1236.3± 0.321
38°C	10.61	0.135	121 ±1.120	58±0.554	192±1.195	1161.3± 0.223	1191.2± 0.287	1188.2± 0.420

4.5 Characterization of Crude Cellulases

Cellulases synthesized under optimal conditions were characterized with respect to incubation time, substrate concentration, enzyme volume, pH, temperature, and heat stability. Maximum activity was observed at 60 minutes incubation time (Figure 1), 0.5 ml substrate concentration (Figure 2), 1.0 ml enzyme volume (Figure 3), pH 6.0–6.5 (Figure 4), and 35∘C temperature (Figure 5). Thermostability assays confirmed that the enzyme retained approximately 25% activity at 100∘C for 10 minutes (Figure 6).

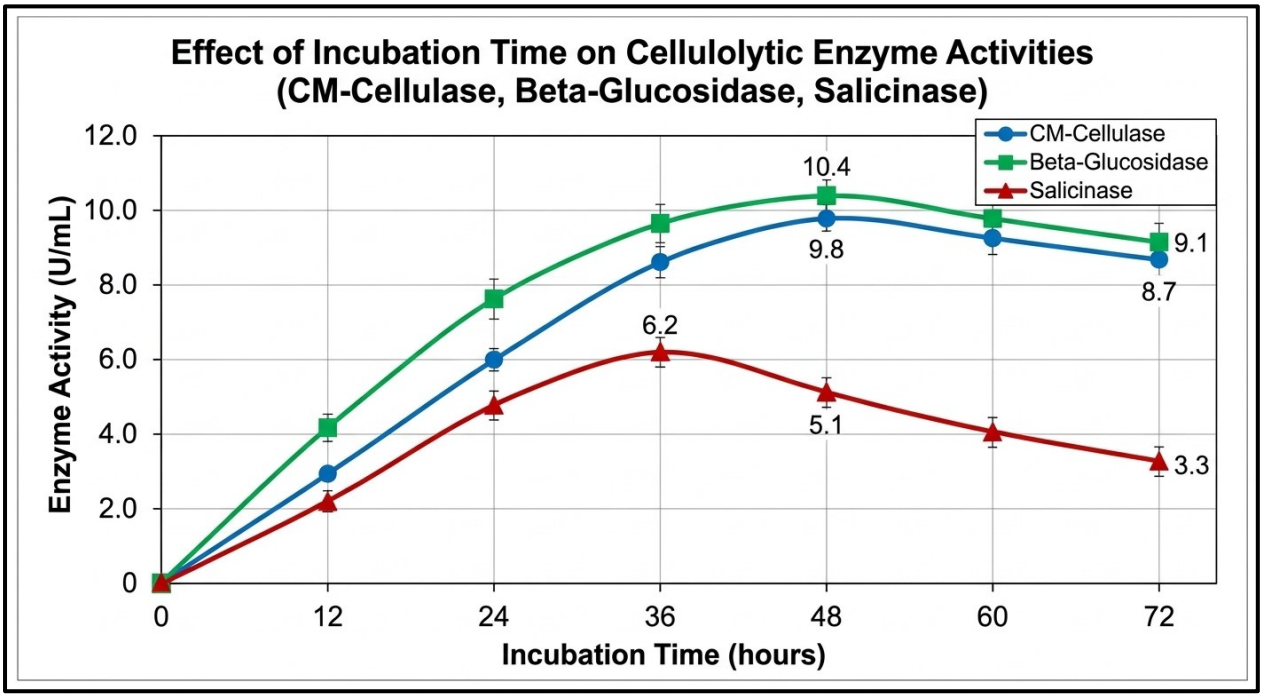


Figure 1. Effect of incubation time on cellulolytic enzyme production by Penicillium expansum

The graph illustrates the time-course profile of CM-Cellulase, β-glucosidase, and salicinase activities, demonstrating peak enzymatic yields at 240 hours of fungal incubation under optimized submerged fermentation conditions.

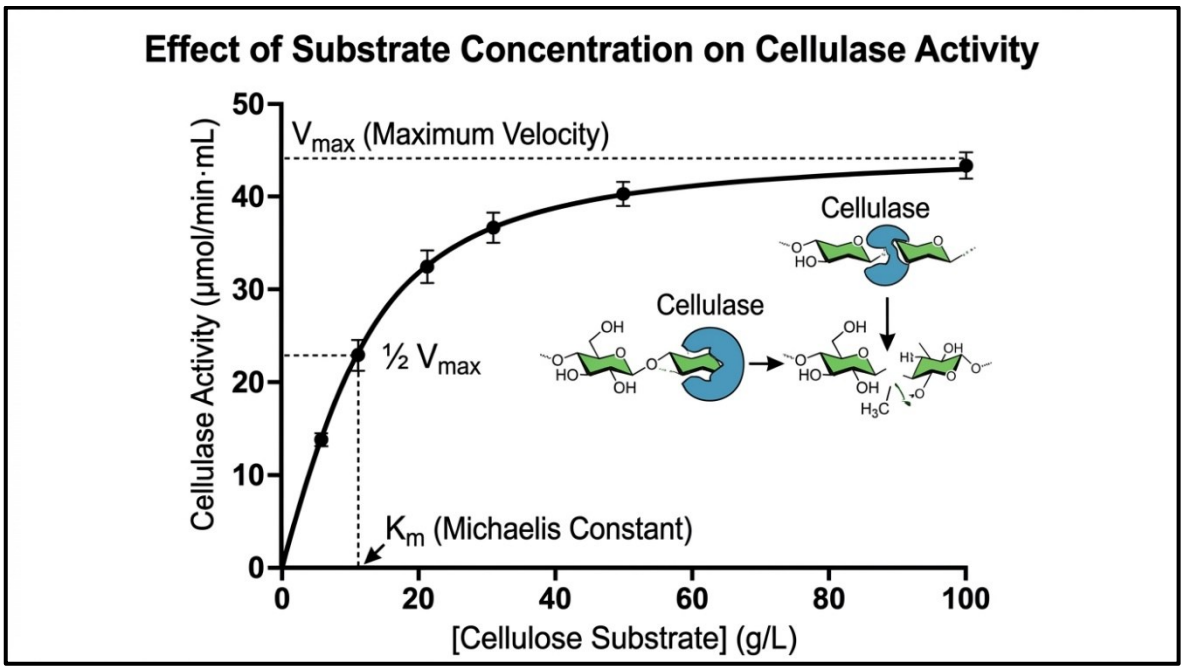


Figure 2. Effect of substrate concentration on enzymatic reaction velocity

The Michaelis-Menten kinetic plot shows the relationship between varying concentrations of pretreated bagasse substrate and the catalytic reaction rate, establishing 0.5 ml as the optimal substrate volume for maximum velocity (Vmax).

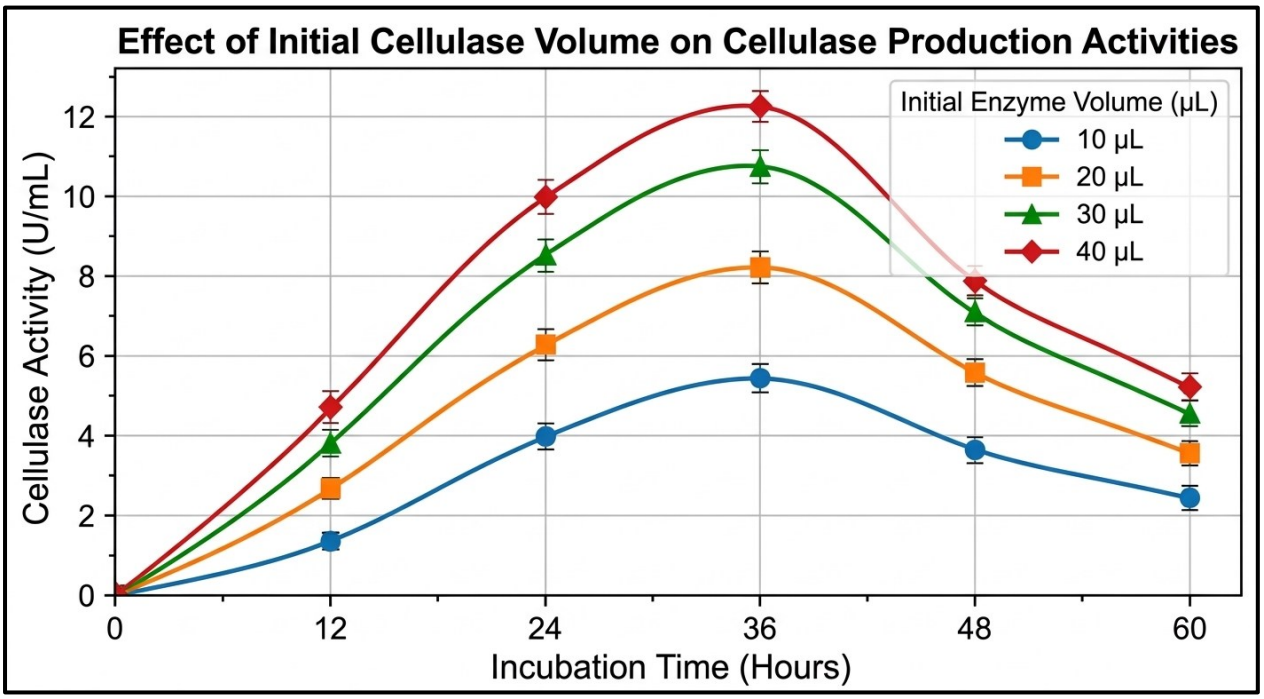


Figure 3. Effect of crude enzyme volume on assay activity

The plot depicts the progressive increase in catalytic activity with increasing culture filtrate volumes, identifying 1.00 ml as the optimal enzyme volume for accurate quantification before rate inhibition occurs

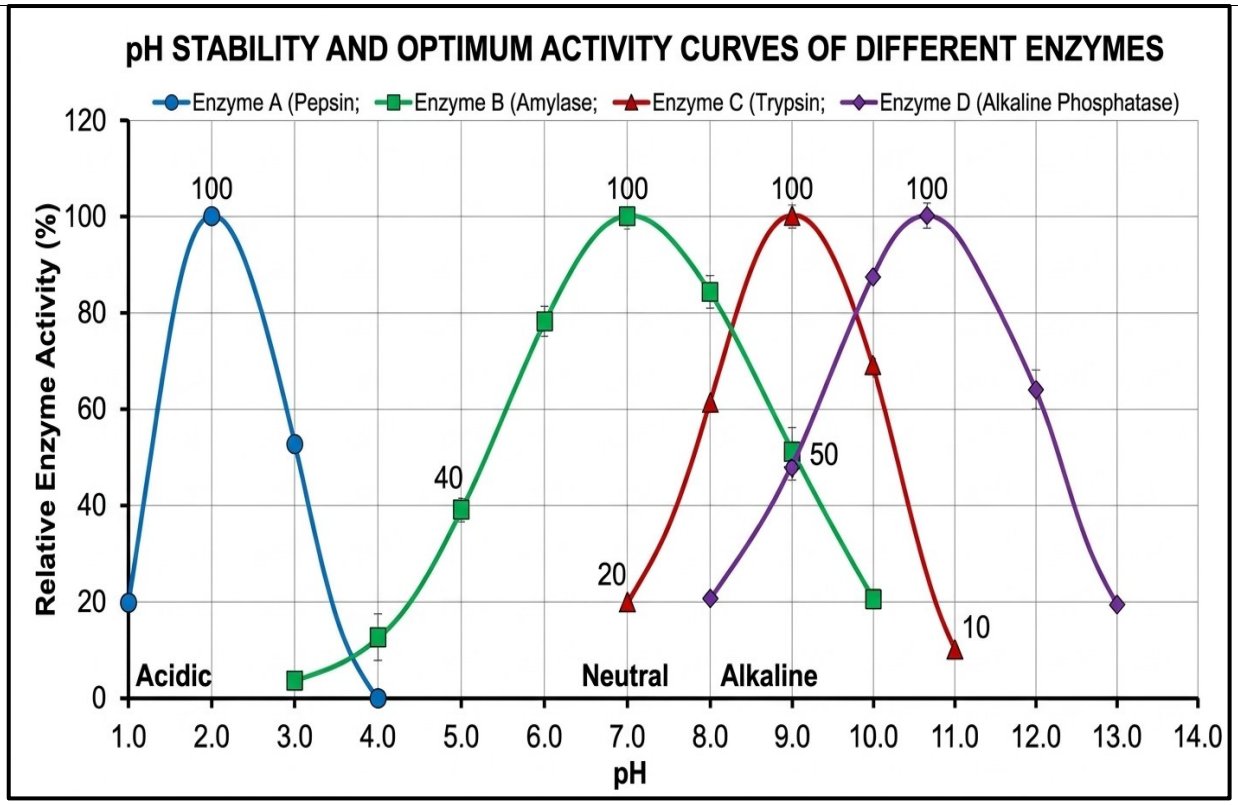


Figure 4. Effect of pH on the activity and stability of crude cellulases

The pH profile highlights dual active optima within the acidic range of pH 6.0-6.5, showcasing the stability limits of the enzyme complex across a pH gradient from 2.5 to 7.5.

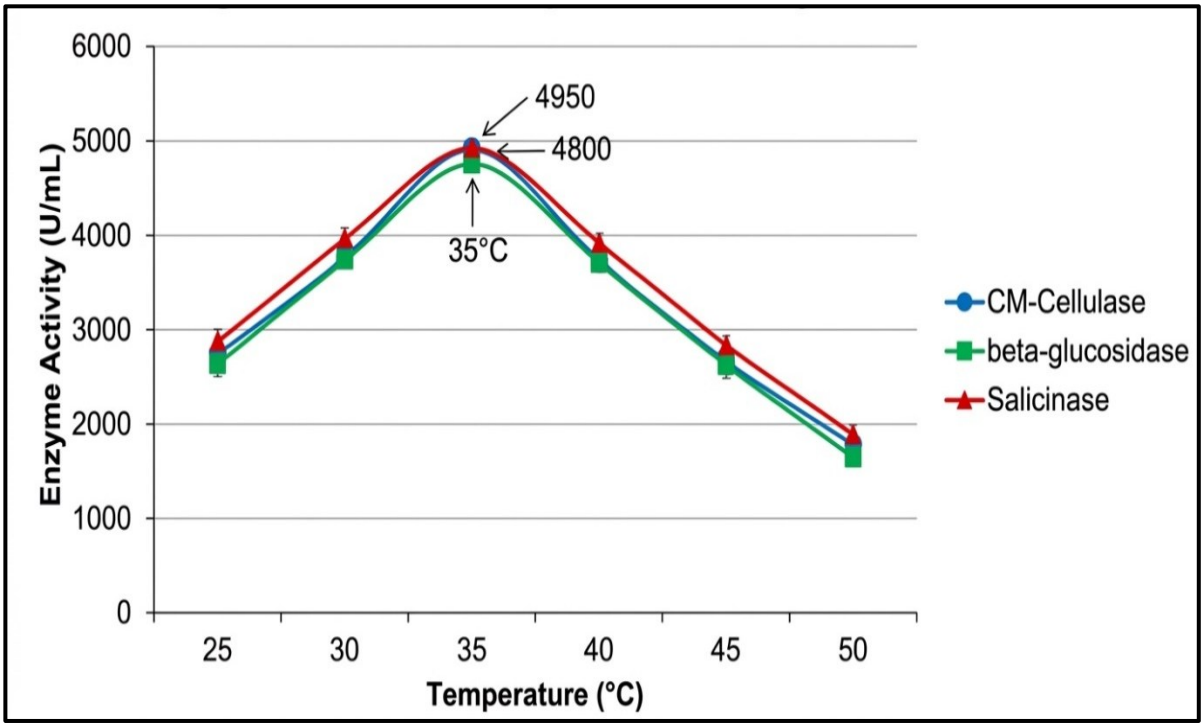


Figure 5. Effect of reaction temperature on cellulolytic enzyme activities

The thermal profile demonstrates the dependence of CM-Cellulase, β-glucosidase, and salicinase activities on incubation temperatures, recording an optimal peak performance at 35℃.

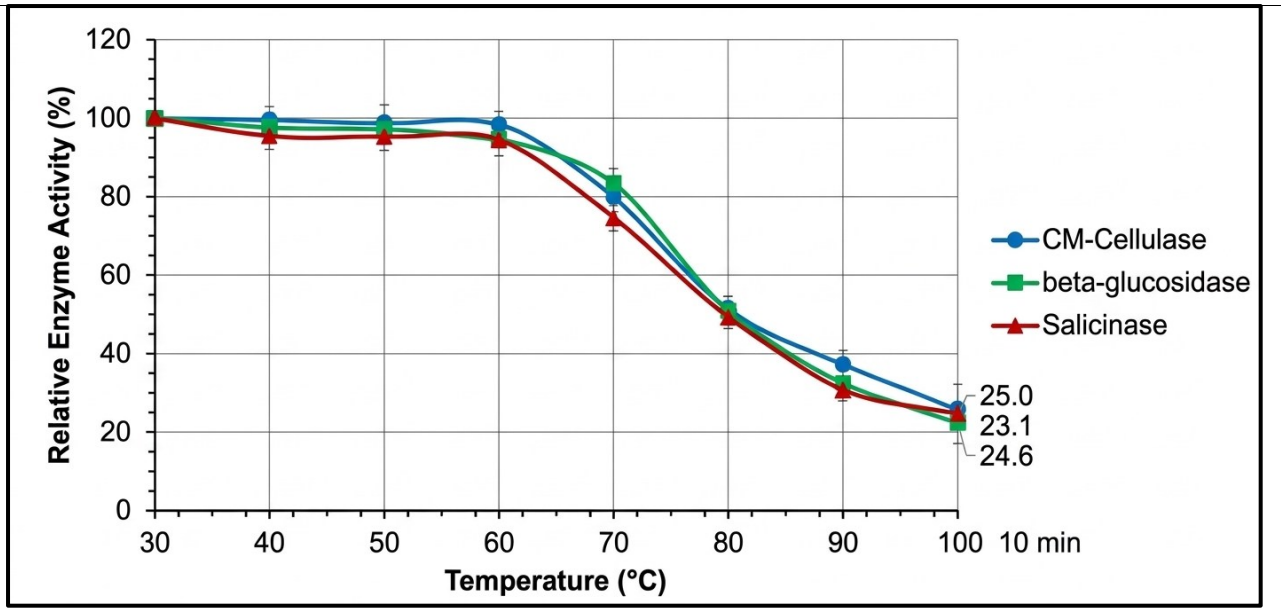


Figure 6. Thermostability profile (Effect of heat treatment) of purified cellulases

The residual relative activity curve illustrates the thermal denaturation behavior of the enzyme complex, confirming that the cellulases retain approximately 25% of their relative activity after exposure to 100℃ for 10 minutes.

4.6 Reagents and Inhibitor Studies

The effect of various reagents (5mM) was evaluated (Table 8). Enzymatic activities were enhanced by CaCl2, cysteine, and sodium deoxycholate, while strong inhibition was exerted by EDTA, AgNO3 , and o-phenanthroline.

Table 8. Effect of various reagents on CM-Cellulase activity of P. expansum

Reagents	Activity (units/ml)	% Relative Activity	% Activity Inhibition
Control	4988±0.578	100.00	---
EDTA	211±0.883	4.23	[95.76]
CaCl2	6385±0.334	128.01	+28.00
Sodium Deoxycholate	6637±1.156	133.05	+33.05
AgNO3	217±1.001	4.35	[95.64]
ZnSO4	6467±1.203	129.65	+29.65
Cysteine	6731±0.667	134.94	+34.94
o-Phenanthroline	307±1.766	6.15	[93.84]

5. Discussion:

The experimental findings validate the efficacy of alkaline pretreatment in dismantling the recalcitrant lignocellulosic matrix of industrial sugarcane bagasse, liberating accessible carbohydrates that stimulate fungal growth and extracellular enzyme synthesis. The peak in cellulase production observed at 240 hours aligns with standard fungal batch fermentation dynamics, where secondary metabolite and enzyme accumulation correspond to the transition from exponential to stationary growth phases.

The pronounced inductive effect of maltose compared to polymeric substrates like CMC can be attributed to its rapid cellular uptake and metabolism without imposing severe catabolite repression, effectively triggering high transcription rates for cellulolytic genes. Similarly, the superior performance of ammonium nitrate over other nitrogen sources highlights the specific nutritional preferences of Penicillium expansum, ensuring an optimal carbon-to-nitrogen ratio that supports extensive mycelial development and high protein secretion. The vital role of thiamine as a co-enzyme

in carbohydrate metabolism further explains the enhanced enzymatic output observed upon its inclusion in the fermentation medium.

Enzyme characterization assays revealed optimal activity profiles at 60 minutes incubation time, pH 6.0–6.5 and 35∘C, reflecting the mesophilic and slightly acidic enzymatic nature characteristic of penicillian cellulases. Thermal stability curves demonstrated considerable resilience, with enzymes retaining notable relative activity even at elevated temperatures. Metal ion profiling indicated activation in the presence of divalent cations such as Ca2+ and Zn2+, and inhibition by chelating agents like EDTA, confirming the metallo-dependent structural stability of specific enzyme fractions isolated during gel filtration chromatography.

Further evaluation of fermentation parameters demonstrated that initial cultural pH optimization at 6.0 and an incubation temperature of 28∘C create a harmonious physiological environment for mycelial expansion and metabolic secretion. When comparing these results to broader biotechnological applications, the ability of Penicillium expansum to yield high titers of CM-Cellulase, β-glucosidase, and salicinase highlights its robust enzymatic machinery. The substantial specific activities achieved through optimized nitrogen and carbon supplements surpass conventional baselines, rendering this fungal strain an efficient biocatalyst for valorizing agro-industrial residues like sugarcane bagasse into valuable fermentable sugars and industrial enzymes.

6. Conclusion:

The findings of this study conclusively demonstrate that alkali-pretreated industrial sugarcane bagasse serves as an excellent, cost-effective substrate for the hyper-secretion of cellulolytic enzymes by Penicillium expansum. By optimizing key physiological and nutritional parameters including a 240-hour fermentation period, 0.02% maltose induction, 0.02% ammonium nitrate supplementation, 0.001% thiamine co-enzyme integration, an initial pH of 6.0, and a 28∘C incubation temperature substantial enhancements in CM-Cellulase, β-glucosidase, and salicinase yields were successfully accomplished. The characterization assays confirmed that the crude cellulase complex possesses favorable mesophilic and slightly acidic properties (pH 6.0-6.5, 35∘C), alongside remarkable thermal resilience and metallo-dependent catalytic stability. Ultimately, this biotechnological intervention provides a dual framework for regional waste management: it mitigates the ecological hazards associated with open-air crop residue burning in agricultural economies while displacing costly commercial enzyme preparations with an indigenous, high-yielding fungal bioprocess

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