



Production, Optimization, and Partial Purification of Cellulases by *Penicillium expansum* Fermented with Industrial Sugar Cane Bagasse

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ABSTRACT

There is an extensive use of biological substances such as enzymes in a variety of industrial and medicinal fields. Cellulase, has gained much interest worldwide as it's a biocatalyst for converting cellulose into simple sugars. The present was designed to explore and develop the renewable energy resources. The bagasse as agro-waste offers as an economical and sustainable substrate for cellulase synthesis. Acidic conditions (pH 6.0) hydrolyzed the bagasse, which was evident from the fungal growth and the considerable enzyme activity. Cellulase yields of up to 3782.5, 3798.5, and 3491.5 units/mL were recorded during a 240-hour incubation period, at a 28°C optimal temperature. To improve the celluloses produced by *P. expansum*, 0.02% maltose and 0.001% thiamine were added to sugar cane bagasse that had been pretreated with 0.6N NaOH in the mineral media. Sephadex G-100 chromatography was used to isolate five fractions and separated through SDS polyacrylamide gel electrophoresis. This study shows that CM-cellulases, β -glucosidase, and salicinase are among the cellulolytic enzymes produced by *P. expansum*. These cellulases have an acidic, neutral, and alkaline pH, according to experimental research. While some cellulases are heat stable and preserve more than 20–25% of their activities at 100°C, others are heat labile and lose about 60% of their activities at that temperature. Cellulase possesses the property of being metalloenzymes, meaning that their active center has both a thio and a sulfhydryl group. Herein, we are reporting the use of bagasse as carbon source for microbes. Ultimately, highlighting the use of microbes in stable and effective production of cellulases or other important biomolecules of biomedical importance.

INTRODUCTION

The rapid increase in the energy problem and the usage of fossil fuels have produced significant pollution in the environment, which has spurred researchers to develop strategies to employ renewable energy resources for energy generation. Lingo cellulosic biomass is a viable substitute for fossil fuels (1). Its main constituents are cellulose, lignin, and hemicellulose; these are the most prevalent, untapped, and indigestible sources of renewable energy for humans (2). In essence, cellulose is a polymer made of glucose monomers joined by β -1,4 glycoside linkages that is insoluble in water. Using commercially accessible technologies or microbes, we may effectively transform these glucose monomers through fermentation into bioethanol and various other compounds with added value (3). Cellulosic bio-waste's complex microcrystalline structure makes it difficult to convert into resources for food, energy, and feed (4). Different established procedures that used mineral acids and alkalis in combination with ionic liquids alongside sub- and supercritical water attempted to defeat the cellulose structural issues for effective hydrolysis.

Traditional procedures cause substantial costs, vessel corrosion problems, and waste disposal complications (5). Researchers propose an environmentally friendly cellulose enzyme complex approach that works as an alternative solution to restrictions while minimizing environmental dangers. A class of glycol hydrolases, including cellobiohydrolase (exo-1,4- β -D-glucanase, EC 3.2.1.91) and endoglucanase (endo-1,4- β -D-glucanase EG, EC 3.2.1.4). That one functions internally on the cellulose chain by cleaving linked bonds and freeing non-reducing ends that eliminate cellobiose from the non-reducing end of the cellulose chain and β glucosidase (1,4- β -D-glucosidase, BG, EC 3.2.1.21), which, taken together, are known as the cellulase enzyme complex, complete the hydrolysis by dividing cellobiose and tiny cello-oligosaccharides to glucose (6).

The cellulase enzyme complex is what breaks down cellulosic biomass into its individual monomeric units in a selective manner. These monomeric units can serve as raw materials for a variety of sectors, including animal feed, bioethanol (7), food, paper, and pulp (8), and chemicals. Numerous varieties of microbes, such as fungi (9), trigger

the synthesis of cellulase enzymes naturally. *Trichoderma* spp. and *Bacillus* spp. are the most researched and used microorganisms for the industrial synthesis of cellulase (10). Cellulase manufacturing economics are primarily hindered by the substrate's intrinsic cost and the enzyme's minimal yield, both of which are detrimental to the use of the enzyme on a large scale. Improving the economics of cellulase enzyme production is possible by using agro residues like wheat bran, which is accessible at low prices compared to cellulose and advice, and it is available with 30% cellulosic content (11). In the same way, we can overcome restrictions on low enzyme yield by adding other appropriate microbial species or stimulating cellulase synthesis by suitable optimization of the parameters regarding the enzyme yield (12).

According to Premalatha et al., we can regulate the production of extracellular enzymes by adjusting the nutritional composition of the medium, which includes carbon sources and mineral salts, as well as the physical process parameters, which include temperature, pH, and incubation time (13). These factors have a major impact on the development of industrial bioprocesses to produce enzymes. Regression analysis and factorial design are used in the RSM statistical experimental design, which aids in assessing the effective parameters and their interactions to determine the ideal circumstances for various variables to maximize the production of enzymes (14). The accumulation of excessive amounts of solid waste, including bagasse from sugarcane, rice husk, sorghum husk, banana fruit stalks, and sugarcane peels, is a global concern, as is the hunt for clean energy, which are worldwide concerns. By using cellulase enzymes to hydrolyze each agro waste material's cellulose content, this study aims to analyze how various agricultural waste products degrade into fermentable sugars like glucose. One hydrolytic enzyme that has been identified from bacteria like *P. expansum* is called cellulase.

Accumulation of excess volume of solid wastes such as sugarcane peeler bagasse, banana fruit stalk, sorghum husk and rice husk and search for clean energy which are global issues. The aim of this study to investigate degradation of different agricultural waste materials into fermentable sugars such as glucose by hydrolyzing the cellulose content of each agro waste materials with cellulase enzymes. Cellulase is a hydrolytic enzyme isolated from microorganisms such as *P. expansum*.

Aims, Objectives and Benefits of Study

The aim is to develop low-cost industrial substrates through agricultural and industrial sugarcane wastes for producing essential industrial enzymes such as cellulases while exploring different fields that utilize these enzymes. Importing cellulases and other enzymes into Pakistan through foreign sources burdens the economy with significant million-rupee expenditures yearly. The agricultural foundation of Pakistan makes it an excellent environment for microorganism-based enzyme production through the fermentation of agrarian waste substrates. Research has already been conducted using locally found fungi from indigenous origins for valuable cellulase production. The fundamental aim and

The main goal of this research was to select cost-efficient media to lessen industrial cellulase production expenses while maximizing energy generation from agro and industrial waste materials.

Agricultural waste and industrial waste streams can replace the costly synthetic medium. Scientific laboratories worldwide are researching to produce cellulases from farm waste materials.

Fungi specifically produce the most preferred cellulases because they require shorter times to grow and release large amounts of enzymes during this period.

MATERIALS AND METHODS

The *P. expansum* species was isolated from the soil of the District Khairpur Mir's and identified in the Shah Abdul Latif University Research Laboratory of Mycology. Czepaks agar was used to maintain the stock culture. *P. expansum* was used to inoculate the sterile slants. To achieve luxuriant growth, the slants were incubated at 27°C after inoculation.

Microorganisms

Minerals, water, air, and organic matter make up the soil. Both forest and agricultural soils frequently include cellulolytic bacteria. It was from soil samples that the pure culture of cellulolytic bacteria was isolated and maintained, as stated by Bautista- (15).

Cultural Methods for Soil Microorganisms

The fundamental composition of soil involves different sorts of bacteria and fungi and their associated ecosystem. Because they are chemoorganotrophic organisms, fungi depend on organic compounds for their carbon and energy source. The microbial population in soil holds noteworthy value because it relates to soil fertility and contributes to element biogeochemical cycling while showing industrial possibilities. The Most Probable Number (MPN) technique, the spread plate count, and the plate count techniques are appropriate for counting microorganisms in soil samples (16).

Isolation of Fungi from the Soil Sample

According to Aziz et al., fungi were extracted from the soil sample using a dilution plate technique (17). The mixture contained one gram of dirt and nine milliliters of sterile distilled water, which was shaken for an hour to achieve the 1:10 dilution. The prepared dilutions included concentrations of 1:50, 1:100, and 1:1000.

Three Czepaks Dox agar petri dish replicates received 1 milliliter of each prepared dilution. Seven days of 29°C temperature incubation took place for freshly inoculated Petri dishes. A complied assessment of grown colonies occurred after incubation before their separation process. Fungal cultures obtained throughout the investigation remained on Czepaks Dox agar medium under 25°C conditions.

Chemicals

The chemicals include CMC BDH cellulose carboxymethyl ether for salicin and cellobiose, while sodium potassium tartrate from E Merck and Sigma Chemicals delivered 3,5-dinitrosalicylic acid. In addition to these analytical-grade reagents other reagents were used.

Culture Medium

A solution of $\text{NH}_4(\text{SO}_4)_2$ 2 g/L and nitric acid 2 g/L along with KH_2PO_4 1.0 g/L and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0 g/L; $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ 0.2 mg/L; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 mg/L; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ 0.1 mg/L as well as thiamine hydrochloride 0.1 mg/L served as Culture Medium. The specified quantity of chemicals went into preparing the culture medium and maintaining its chemical integrity. The culture medium reached a pH of 6.0, as per (18). To prepare the spore suspension *P. expansum* received 10.0 ml of sterile water in which the sterile wire loop gently stroked the surface. The spore solution required additional water dilution using sterilized water, making it reach 100 milliliters (19).

Hydrolysis of Agricultural Waste

The slurry received 800 ml of 0.6N NaOH solution to hydrolyze 10.0 g of rice husk, banana fruit stalks, sugarcane peeler bagasse, and industrial sugarcane bagasse under flame heat for two hours while maintaining constant slurry height. The autoclaved successful slurry received a pressure treatment of 1.5 kg per cm^2 for thirty minutes. Slurry solutions reached the surrounding temperature before filtering through man No. 1 filter paper. The agricultural waste solution obtained from filtration underwent subsequent use as a carbon source in mineral media.

The researcher incorporated the carbon source from agricultural waste into a mineral media solution. The agricultural waste material was dried under an oven set at 105°C to obtain a constant weight, from which weight loss calculations were conducted (20).

Condition for Cultivation

Experimentation took place in 250 ml conical flasks containing cotton wool among 50 ml of solubilized agricultural waste mixed with mineral media, which underwent autoclaving under 1.5 kg/cm^2 pressure for 20 minutes. *P. expansum* spores received a one-milliliter addition into a sterile medium once they reached room temperature. The shaking incubator operated with 200 rpm at $28 \pm 2^\circ\text{C}$ to incubate the flasks containing cooled orbital rotations. The mycelial culture broth was extracted from the mycelia using filter paper known as man No. 1 after the 24-hour incubation. Examinations were conducted on β -glucosidase and CM-cellulase activities with the culture broth material. The scientists dried the mycelium until its weight became stable through 105-degree Celsius oven heating procedures (21).

Effect of Culture Conditions

Effect of Incubation Time on Synthesis Cellulase

The study of ideal growth duration for cellulase production revealed important findings through the traditional incubation method as per the cultivation parameters description. The studies about cellulase activities covered a time frame from 24 to 240 hours. Enzyme synthesis reaches its maximum point at a particular time frame which depends on the used agricultural waste hydrolysis technique and microbial species and carbon source kind. The selected microorganism *P. expansum* grew in a media mixture that used a mineral medium combined with 0.6N NaOH-treated

industrial sugarcane bagasse filtrate. The synthesis of the enzyme reached its maximum quantity at 240 hours.

Effect of Different Carbon Sources on the Synthesis of Extracellular Cellulases

Cells of *P. expansum* use different carbon sources, such as sugarcane bagasse hydrolysate and its eight pure components, to produce extracellular cellulases. The culture medium received pure sugars through aseptic addition after each sugar underwent its separate sterilization process. During the ten days at $28 \pm 2^\circ\text{C}$, the flasks with inoculation remained in a cold orbital shaking incubator.

Effect of Different Nitrogen Sources on the Production of Extracellular Cellulases

The standard method requires using yeast, ammonium chloride, urea, albumin, casein, and peptone instead of $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source in the mentioned fermentation medium.

Impact of Distinct Vitamins on the Production of Cellulase

Following a conventional experimental procedure, researchers tested ascorbic acid and vitamin D2 along with riboflavin and β -Carotene and nicotinic acid and nicotinamide and pyridoxine at identical concentrations as vitamin thiamine in the culture media for cellulase production. The cellulase production showed maximum results using vitamin thiamine when other vitamins were evaluated.

Cellulase production peaked when beginning culture media preparations at different pH levels ranging from 4 to 10. Before sterilizing the media, the author adjusted pH levels with HCl or NaOH chemicals. Both inoculation and incubation periods employed the conventional method of execution. When the starting pH reached 6.0, maximum production rates were achieved for cellulase enzymes, cellobiose, and salicinase.

Effect of Temperature on Synthesis of Cellulases

P. expansum spore suspension was used to inoculate industrial sugarcane bagasse hydrolysate mineral medium, which was then incubated at various temperatures from 23 to $38 \pm 2^\circ\text{C}$ for 240 hours. CM-Cellulase, cellulose, and Salicinase synthesis reached their highest points when the incubation occurred at $28 \pm 2^\circ\text{C}$.

Assay for CM-Cellulase Activity

The authors employed the method outlined in (22) to evaluate CM-cellulase activity by combining 2.0 ml sodium acetate buffer at pH 4.6 and equally 1.0 ml 1% CM-cellulose with 1.0 ml enzyme sample culture broth. The reaction took place for one hour at 35°C . The dinitro salicylic acid method determined the measurements of released reduced sugar amounts. The CM-cellulase activity measurement depends on analyzing the glucose standard graph to assess results (Figure 1).

Under assay conditions, the amount of CM-cellulase that releases one mg/ml of reducing glucose from CM cellulose is one unit of -CM-cellulase activity.

Assay for β -Glucosidase Activity

A one-milliliter enzyme solution containing the culture broth was mixed with 1.0 milliliters of 1% cellobiose or

salicin solution at 35 °C in two milliliters of pH 4.6 sodium acetate buffer for one hour. Under standard assay conditions, the enzyme concentration required for one milligram of up to one milliliter of glucose production from cellobiose or salicin is considered a unit of cellobiase and salicinase activity (23).

Determination of Total Carbohydrate

Glucose served as the standard material, while the phenol sulfuric acid method established the carbohydrate amounts present in hydrolysate and agricultural waste culture broth. Researchers used Figure 2 as their standard glucose curve to determine the results (24).

Determination of Reducing Sugars

The hydrolysate from agricultural waste and the culture broth underwent reducing sugar analysis using the DNS method and a standard glucose curve to determine their sugar concentrations following (25) (Figure-3).

Total Protein Determination

Employed the total protein determination method to evaluate the culture broth protein content. Bovine serum albumin was the standard for finding the experimental results using a graph (Figure 4). The protein purification process demanded an absorbance measurement at 280 nm to proceed (26).

RESULTS AND DISCUSSION

The extracellular cellulase complex obtained through ammonium sulphate precipitation and gel purification reached homogeneity from *P. expansum* culture filtrate grown on Czapek- Dox medium. The precipitation of cellulase proteins at 40% ammonium sulphate concentration led to the highest activity in all CM-cellulase, β -glucosidase and salicinase enzymatic proteins. The entire purification protocol appears in (Table 1).

Table 1

Isolation and Purification of Cellulolytic Enzymes from Culture Broth of P. expansum.

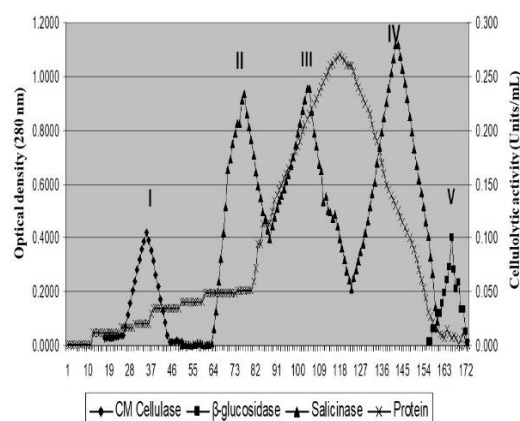
Solvents	Specific activity (units/mg)	Protein mg	% Yield
Crude	11.38	440	100
Act	5.76	295	33.92
MeOH	6.26	290	36.24
20% (NH ₄) ₂ SO ₄	9.16	315	57.63
40% (NH ₄) ₂ SO ₄	10.78	355	76.39
60% (NH ₄) ₂ SO ₄	8.55	320	53.65
80% (NH ₄) ₂ SO ₄	8.64	320	55.20
Dialysis	31.90	271	61.59
F-1	4.93	217	19.10
F-2	1.08	211	4.55
F-3	1.14	210	4.80
F-4	1.18	208	4.92
F-5	2.69	219	11.04

Act: acetone; F-1: fraction-1; F-2: fraction-2; F-3: fraction-3; F-4: fraction-4; F-5: fraction-5; MeOH: methanol;

The elution pattern purification of *P. expansum* cellulases and Sephadex G100. Experimental results in Figure 1 show that CM-cellulase activity appears in fraction 1 of peak 1, whereas peak 2 of fractions 2,3 and 4 possess salicinase activity, and fraction 5 of peak 5 contains B-glucosidase activity. The CM-cellulase activity appears as peak-1 within the fraction 25-46. Peak-2 of fractions 47-91 shows

salicinase activity. The enzyme activity of salicinase was detected in the 92-123 range of peak 3, and peak 4 encompassed the 124-162 range of activity. The fraction 163-172 of peak 5 shows -glucosidase activity.

Figure 1

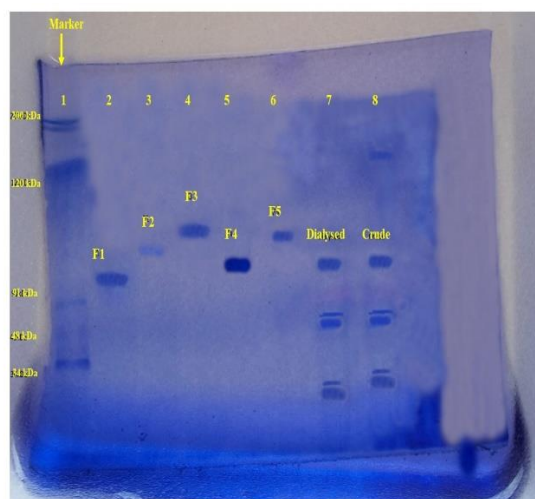


Elution profile of cellulolytic enzymes of *P. expansum* through sephadox G-100 gel filtration by measuring optical density at the wavelength of 280nm. Where, I: CM cellulase, II:-, III:-, IV:-Salicinase, and V: β -glycosidase.

The homogeneity of peak-1, 2, 3, 4 and 5 fractions were determined by SDS polyacrylamide disc gel electrophoresis before the researchers conducted basic enzymatic property tests.

The analysis demonstrates that *P. expansum* makes CM-cellulase, β -Glucosidase and salicinase part of its cellulase aggregate. SDS disc gel electrophoresis confirmed the similarity between fractions while showing single bands in the plate (Figure 2).

Figure 2



Electrophoresis pattern of cellulolytic enzymes. Where, 1: Marker, 2: F1P1 CM cellulase, 3: F2P2 Salicinase, 4: F3P3 Salicinase, 5: F4P4 Salicinase, 6: F5P5 β -Glucosidase, 7: dialysed, 8: crude

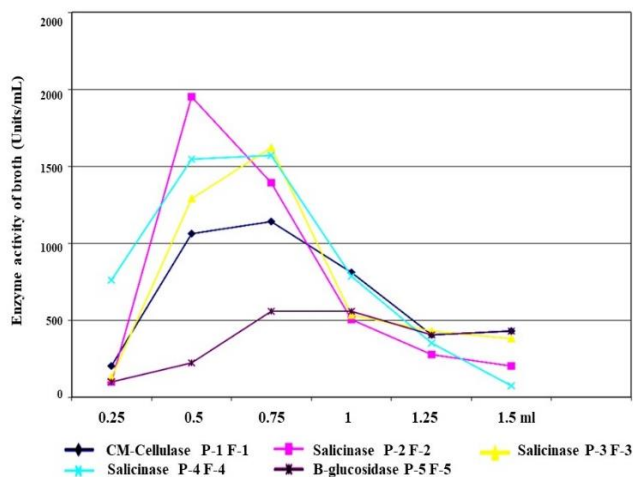
The pooled fraction containing peaks 1, 2, 3, 4, and 5 underwent basic enzymatic tests after its homogeneous structures were determined through SDS polyacrylamide disc gel electrophoresis.

The study on purified cellulose complex reaction velocity under varying substrate levels employed a 0.50 ml purified

enzyme sample with 0.50 ml substrate solutions of different concentrations. The assessment showed that the reaction rate progressed until 0.5 ml CM-cellulase and 0.75 ml β -glucosidase and salicinase were added, as demonstrated in Figure 3, where high substrate concentration decreased reaction speed.

Figure 3

Effect of Substrate Concentration on Purified Cellulases Activities of *p. Expansum*



Studies on Sephadex G-100 separated enzymes with one protein band activity confirmed their characterization through tests of pH values and temperature dependence, as well as thermo-stable effects and inhibitor influences. The peak-1 CM-cellulose exhibited salicinase activity at pH 4.2, while peak-2, peak-3, and peak-4 worked at pH 5.0, 6.0, and 7.5, respectively, for salicinase activity, and peak 5 had β -glucosidase activity at pH 8.0.

The research investigated activity changes within the temperature range of 10–60°C for CM-cellulase peak 1, spiciness peak 2, 3, and 4 β -glucosidase peak 5, where each peak was assessed separately, as displayed in Figure 5. The research findings demonstrated that the optimal temperature existed at 25°C, 35°C, and 40°C, respectively. A research-based investigation of the thermal stability effect focused on purified CM-cellulase peak one, followed by studies of salicinase peaks 2, 3 and 4 and β -glucosidase peak 5 under these conditions.

An enzyme solution took ten minutes within the 20–100±2°C temperature range. The enzyme activity check took place after the sample cooled down utilizing standard assay parameters. The studied CM-cellulase peak 1 and peak 2, 3, and 4 salicinase exhibited less than 5% remaining activity at 80°C while β glucosidase activity of peak 5 maintained 20% and 40% activity at 90°C, thus proving the heat stability of the cellulase complex enzymes.

The research analyzed the outcome of using 5 mM concentrations of different metal ions and reagents on the CM-cellulase peak 1 active component. Table 2 verifies that a-phenanthroline, triton X100, and CaCl₂ produce elevated enzyme activities, though CaCl₂ provides only moderate results. Salicinase function decreases in the presence of EDTA and other substances at a 5-millimolar concentration, such as sodium deoxycholate AgNO₃, ZnSO₄, cysteine, Mercaptoethanol, tween-80, and others.

Table 2

Effect of Various Reagents on Purified p-1 f-1 cm-cellulase Activity of *P. expansum* when Reaction Mixture was Incubated at pH 6.00 at Temperature 35°C for One Hour.

Reagents	Activity	% R. Activity	Activation (%)	Inhibition (%)
Control	1071	100	-	-
AgNO ₃	556.33	51.94	-	48.06
CaCl ₂	1361.00	127.07	27.07	-
Cholic acid Sodium Salt	550.70	51.41	-	48.59
Cysteine	318.98	29.31	-	70.69
C ₂₄ H ₃₉ NaO ₄	201.69	18.83	-	81.17
C ₂ H ₆ OS	201.69	18.83	-	81.17
EDTA	56.12	5.23	-	94.77
Indole acetic acid	100.45	9.37	-	90.63
Mercuric nitrite	67.38	6.29	-	93.71
o-Phenanthroline	2127.81	198.67	98.67	-
SDS	100.45	9.37	-	90.63
Triton X100	201.69	18.83	81.17	-
Tween-80	121.24	11.88	-	88.12
ZnSO ₄	416.51	38.33	-	61.67

To investigate the impact of different reagents on the enzyme activity, they were treated at the concentration of 5 mM. AgNO₃: silver nitrate; C₂₄H₃₉NaO₄: Sodium deoxycholate; C₂H₆OS: Mercaptoethanol; CaCl₂: calcium chloride; EDTA: Ethylenediaminetetraacetic acid; SDS: Sodium dodecyl sulfate; ZnSO₄: zinc sulphate;

Cysteine and Mercaptoethanol slightly boosted metal ions and reagents activity of salicinase (Table-3), but EDTA, CaCl₂, sodium deoxycholate, AgNO₃, ZnSO₄, tween-80 weakened peak-2 salicinase function. SDS, phenanthroline, Cholic acid Sodium Salt, Mercuric nitrite, Triton X100, and Indole acetic acid show inhibition effects. The impact of various metal ions and reagents on salicinase activity. The data confirms that sodium deoxycholate and tween-80 enhance enzyme performance, but our findings show this in Table 4.

Table 3

Effect of Various Reagents on Purified p-2 f-2 Salicinase Activity of *P. Expansum* when Reaction Mixture was Incubated at pH 6.00 at Temperature 35°C for One Hour.

Reagents	Activity	% R. Activity	Acti. (%)	Inh. (%)
Control	590	100	-	-
AgNO ₃	284.85	48.27	-	51.73
CaCl ₂	48.47	8.21	-	91.79
Cholic acid Sodium Salt	194.29	32.93	-	67.07
Cysteine	650	110.16	10.16	-
C ₂₄ H ₃₉ NaO ₄	96.96	16.43	-	83.57
C ₂ H ₆ OS	675	114.40	14.40	-
EDTA	45.21	9.18	-	90.82
Indole acetic acid	48.47	8.21	-	91.79
Mercuric nitrite	42.41	7.18	-	92.82
o-Phenanthroline	103.02	17.46	-	82.54
SDS	248.34	42.09	-	57.91
Triton X100	89.72	15.21	-	84.79
Tween - 80	70.96	12.02	-	87.98
ZnSO ₄	177.55	30.09	-	69.96

To investigate the impact of different reagents on the enzyme activity, they were treated at the concentration of 5 mM. AgNO₃: silver nitrate; C₂₄H₃₉NaO₄: Sodium deoxycholate; C₂H₆OS: Mercaptoethanol; CaCl₂: calcium chloride; EDTA: Ethylenediaminetetraacetic acid; SDS: Sodium dodecyl sulfate; ZnSO₄: zinc sulphate; Acti: Activity; Inh: Inhibition;

Table 4

Effect of Various Reagents on Purified p-3 f-3 Salicinase Activity of *P. Expansum* when Reaction Mixture was Incubated at pH 6.00 at Temperature 35°C for One Hour.

Reagents	Activity	% R. Activity	Activation (%)	Inhibition (%)
Control	2426	100	-	-
AgNO ₃	1378.38	56.81	-	43.19
CaCl ₂	150.08	6.18	-	93.82
Cholic acid Sodium Salt	3445	142.00	42.00	-
Cysteine	1024.90	42.24	10.16	57.76
C ₂₄ H ₃₉ NaO ₄	4739.43	195.35	95.35	-
C ₂ H ₆ OS	743.79	30.65	14.40	69.35
EDTA	200.04	8.24	-	91.76
Indole acetic acid	128.35	5.29	-	94.71
Mercuric nitrite	150.08	6.18	-	93.82
o-Phenanthroline	399.89	16.48	-	83.52
SDS	200.04	8.24	-	91.76
Triton X100	414.70	17.09	-	82.91
Tween - 80	289.59	11.93	88.077	-
ZnSO ₄	1084.24	44.69	-	55.31

To investigate the impact of different reagents on the enzyme activity, they were treated at the concentration of 5 mM. AgNO₃: silver nitrate; C₂₄H₃₉NaO₄: Sodium deoxycholate; C₂H₆OS: Mercaptoethanol; CaCl₂: calcium chloride; EDTA: Ethylenediaminetetraacetic acid; SDS: Sodium dodecyl sulfate; ZnSO₄: zinc sulphate; Salicinase experiences strong activation with specific activators such as EDTA and marked inhibition from the tested substances. The enzyme activation response for Cholic acid Sodium Salt lies between the results of the tested activators.

The research analyzed what various reagents and ions do to the activity of salicinase peak-4. Table 5 shows enzyme activation occurs at an average level between CaCl₂ and ZnSO₄ plus cysteine and mercaptoethanol tests. Only EDTA Sodium deoxycholate and the following chemicals inhibit enzyme functioning.

Table 5

Effect of Various Reagents on Purified p-4 f-4 Salicinase Activity of *P. Expansum* when Reaction Mixture was Incubated at pH 6.00 at Temperature 35°C for One Hour.

Reagents	Activity	% R. Activity	Activation (%)	Inhibition (%)
Control	2839	100	-	-
AgNO ₃	1728.64	60.86	-	39.14
CaCl ₂	3851	135.64	35.64	-
Cholic acid Sodium Salt	195.79	6.89	-	93.11
Cysteine	3348	117.92	17.92	-
C ₂₄ H ₃₉ NaO ₄	587.37	20.68	-	79.32
C ₂ H ₆ OS	3785	133.32	33.32	-
EDTA	163.07	5.74	-	94.26
Indole acetic acid	130.48	5.59	-	94.41
Mercuric nitrite	260.97	9.19	-	90.81
o-Phenanthroline	554.66	19.53	-	80.47
SDS	195.79	6.89	-	93.11
Triton X100	460.11	16.21	-	83.80
Tween - 80	482.91	17.00	-	83.00
ZnSO ₄	3571	125.78	25.78	-

To investigate the impact of different reagents on the enzyme activity, they were treated at the concentration of 5 mM. AgNO₃: silver nitrate; C₂₄H₃₉NaO₄: Sodium deoxycholate; C₂H₆OS: Mercaptoethanol; CaCl₂: calcium chloride; EDTA: Ethylenediaminetetraacetic acid; SDS: Sodium dodecyl sulfate; ZnSO₄: zinc sulphate; Researchers studied what metal ions and reagents change the activity of purified β-glucosidase peak-5. Tests in Table

6 prove that CaCl₂, ZnSO₄, and Mercaptoethanol accelerate beta-glucosidase enzyme reactions. The enzyme shows a fast decline in effectiveness even with low amounts of SDS and Sodium deoxycholate alongside AgNO₃, Cysteine, Tween-80, o-phenanthroline, Cholic acid Sodium Salt, Mercuric nitrite, Triton X100, and Indole acetic acid.

In case of purified cellulases these results are favorably compared with the findings of other workers synthesized from different sources (27).

Table 6

Effect of Various Reagents on Purified P-5 F-5 β-Glucosidase Activity of *P. expansum* when Reaction Mixture was Incubated at pH 6.00 at Temperature 35°C for One Hour.

Reagents	Activity	% R. Activity	Activation (%)	Inhibition (%)
Control	2376	100	-	-
AgNO ₃	1265	53.24	-	46.76
CaCl ₂	2695	113.42	13.42	-
Cholic acid Sodium Salt	1632.25	68.69	-	31.31
Cysteine	2176.34	91.59	-	8.41
C ₂₄ H ₃₉ NaO ₄	407.44	17.14	-	82.86
C ₂ H ₆ OS	1845.16	77.65	-	22.35
EDTA	1197.93	50.41	-	49.59
Indole acetic acid	71.91	3.02	-	96.98
Mercuric nitrite	913.11	38.43	-	61.57
o-Phenanthroline	407.44	17.14	-	82.86
SDS	167.76	7.06	-	93
Triton X100	364.30	17.14	-	82.86
Tween - 80	505.76	21.28	-	78.72
ZnSO ₄	2680.68	112.79	12.79	-

To investigate the impact of different reagents on the enzyme activity, they were treated at the concentration of 5 mM. AgNO₃: silver nitrate; C₂₄H₃₉NaO₄: Sodium deoxycholate; C₂H₆OS: Mercaptoethanol; CaCl₂: calcium chloride; EDTA: Ethylenediaminetetraacetic acid; SDS: Sodium dodecyl sulfate; ZnSO₄: zinc sulphate.

CONCLUSION

Present study showed that agro-wastes including bagasse, which can be resourcefully exploited as alternative carbon source for growth of microbes ultimately microbial mediated production of important enzymes such as cellulase. The results demonstrated that *P. expansum* variety of cellulases having diverse features. Further Using column chromatography (Sephadex G-100) and SDS gel electrophoresis five fractions for CM-cellulase, salicinase, and β-glucosidase were observed. Purified *P. expansum* cellulases have an optimal pH that is acidic, neutral, or alkaline. These enzymes are heat stable, and research indicates that some enzymes lost about 60% of their enzymatic activity at 100°C, while a small percentage of the enzymes preserved 15-20% of their activity at that temperature. According to the current research, salicinase purified fractions' 60-70°C temperature range maintains 50% of the enzyme's activity. Remarkably, retaining 20% of activities at 100°C of salicinase and CM-cellulase exhibiting prominent heat stability.

Furthermore, metal ions also influence on the enzymatic activities. Such as CaCl₂ induce the activities of β-glucosidase and CM-cellulase. While, o-phenanthroline and EDTA suppressed their activities signifying them as metalloenzymes. Besides, heavy metals like ZnSO₄ and AgNO₃ inhibits the β-glucosidase, salicinase and CM-

cellulase while mercaptoethanol and cysteine increased showing the presence of thiol and sulfhydryl groups at their active sites. Out and out, the current study exhibiting the potential of *P. expansum* produced cellulases with distinct properties. Which make it a promising organism

for the production of efficient and stable cellulase enzymes for biomedical industry.

Ethical Approval: The study was approved by local research ethics committee.

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