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Cellulase production from *Bacillus spp*. and purification with activated charcoal

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Abstract

Many bacteria have demonstrated the potentials of producing cellulases in copious amount. Bacteria of the genus *Bacillus* can utilize cheap lignocellulosic biomass to produce many bioproducts; like cellulases. One of the drawbacks in utilizing bacteria for the industrial production of cellulases is the high cost of recovering the enzyme from a submerged fermenting medium. This study seeks to produce cellulase from rice husk in a submerged fermentation. Species of Bacillus were isolated from cellulosic dumpsites, characterized and screened qualitatively for cellulase production on Carboxymethylcellulose (CMC) agar plates. The organisms were grown on mineral salt basal medium ($NaNO_3$- 2g, K_2HPO_4-1g, $MgSO_4$-0.5g, KCl-0.5g, CMC-2g, peptone-0.2g and agar- 8.5g), incubated at 40°C. The optimum conditions for the production of cellulase was found to be 60°C, at pH of 5.5 and substrate concentration of 1.0% (w/v). The crude enzyme was subjected to activated charcoal purification and the cellulase showed a 2.5 (with *B. megaterium*) and 1.4-fold purification (with *B. cereus*) in one -step purification with (3% w/v) activated charcoal, at temperature of 50 °C and pH (6.0) and contact time (3hrs). The result of SDS-PAGE analysis of purified cellulase using silver staining techniques showed four major bands corresponding to molecular weights of 30, 42, 55 and 70 kDa. The use of *Bacillus* sp. in downstream processing of lignocellulosic biomass pretreatment and activated charcoal in cellulose purification offers a huge opportunity for cutting cost. This method of enzyme purification is inexpensive, rapid, and simple which could facilitate downstream processing of industrial enzymes.

Keywords: Cellulase; *Bacillus Spp*; Activated Charcoal; Silver Staining

1. Introduction

Cellulose is the substrate for cellulase pretreatment. Agricultural wastes are rich sources of lignocellulosic biomass and are the most abundant in nature. Cellulases are a complex group of enzymes which are secreted by a broad range of microorganisms including fungi, bacteria, and actinomycetes. The use of microorganisms or their enzymes in industrial processes is an ecofriendly and cheap approach to lignocellulosic biomass pretreatment.

This approach is gaining importance as it can be performed under mild conditions, consumes less energy and generates non-toxic bye products, thus offering an environmentally safe process (Zhang et al. 2020).

The high cost of microbial enzymes has been attributed to the substrates used (Majidi et al., 2021), which therefore has created an impediment in the use of enzymes for industrial processes.

Several species of Bacillus have been studied and reported for their copious amount and efficient hydrolytic activity, (Malik et al., 2021). Microorganisms screened from the environment possess properties like being thermophilic or

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psychrophilic, acidophilic or alkaliphilic. This wide range of diversity obtained by microbes helps out in screening the efficient cellulase producing bacteria to overcome the challenges associated with industrial processes. (Sarwan et al., 2024).

Enzyme production involves various steps such as selection of suitable organism, screening for enzyme production, fermentation; which could be solid-state fermentation (SSF) or submerged fermentation (SmF) protein separation and purification.in SmF, free-flowing liquid substrates like molasses and broths are used.

The end products of the fermentation are liberated into the fermentation broth. Substrate utilization is very rapid in SmF; hence substrate must be provided continuously for this fermentation process.

Microorganisms screened from the environment possess properties like being thermophilic or psychrophilic, acidophilic or alkaliphilic. This wide range of diversity obtained by microbes helps out in screening the efficient cellulase producing bacteria to overcome the challenges associated with industrial processes. (Singh, 2017). Enzyme production involves various steps such as selection of suitable organism, screening for enzyme production, fermentation; which could be solid-state fermentation (SSF) or submerged fermentation (SmF) protein separation and purification.in SmF, free-flowing liquid substrates like molasses and broths are used. The end products of the fermentation are liberated into the fermentation broth. Substrate utilization is very rapid in SmF; hence substrate must be provided continuously for this fermentation process.

This technique is well suited for secondary metabolites from bacteria because it requires high moisture content for their growth. SmF has several advantages in which genetically modified organisms are grown well compared to SSF.

Further, process parameters such as pH, temperature, moisture, oxygen transfer and aeration can be controlled. (Yimer and Tilahun, 2018).

Activated charcoal is an adsorbent widely used in the treatment of wastewater and industrial contaminants by virtue of its high removal capacity and adaptability for wide range of pollutants. It is made from essentially carbonaceous materials; tree bark, coal, cotton waste, palm kernel shell, and many agricultural by-products and their ability to remove colours have been reported. Although, application of activated charcoal in the decolorization of enzyme converted glucose syrup had been reported, its uses in the purification of microbial enzymes have been scanty (Kareem, et al., 2011).

2. Materials and Methods

Cellulolytic bacteria of the genus Bacillus were isolated from cellulosic sites (termitarium, cassava peel dump site, palm oil mill effluent site, and saw mill dumpsite) as described by (Malik et al. 2020).

Culture media including Nutrient agar and Carboxymethyl cellulose (CMC) agar were used. All media were prepared in accordance with the manufacturers' specifications. 28g of Nutrient agar (NA) and 48g Carboxy Methyl Cellulose (CMC) agar were separately dissolved in 1000ml of distilled water in flasks, the flasks were heated in a hot plate, the media were sterilized by autoclaving at 121°c for 15 minutes, they were each allowed to cool to about 45°C before they were aseptically poured in 15ml portion into petri dishes where they were allowed to cool. The sterile solid plates were used for the microbial culture.

A point inoculation of isolated bacteria was carried out on CMC agar plates, incubated at 37^{o}C for 24hours. The plates were flooded with iodine solution for 2 minutes and rinsed with distilled water. Zones of clearance were measured and taken as cellulolytic activity, the organisms which displayed zones of clearance were used for further studies.

The quantitative screening for cellulase activity was measured by the amount of reducing sugars released per ml of sample per minute under assay condition. Endoglucanase and Exoglucanase activities were determined by 3,5-Dinitrosalicylic acid method (DNS) (Miller 1959) using CMC and Filter Paper (FP) as substrates, respectively (Zhang et al., 2020).

Of the ten bacteria isolated, four were prospective cellulolytic bacteria, two of the four bacteria were selected for enzyme production. The culture broth obtained from the 72-hour incubation was centrifuged at 5000 rpm at 4^{o}C for 15 minutes to separate the bacterial cells from the supernatant. The supernatant was mixed with 4% (w/v) of activated charcoal (pH 4.5), as described by Kareem et al., 2011, incubated in a water bath at 50^{o}C and stirred occasionally for 30 minutes. The mixture was allowed to cool, centrifuged at 5000 rpm at 4^{o}C and the supernatant carefully decanted.

The partially purified enzyme was rechecked for cellulase activity and the protein content was estimated by Biuret method (Gornal et al., 1949). The molecular weight of partially purified cellulase was determined by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Yu and Li 2015. The molecular ladder (10-245 kDa) (Thermo Fisher Scientific) was used to determine the molecular weight of partially purified enzyme. After the run at 70V for 90 minutes, the gels with protein markers were stained with silver stain (24600 Thermo Fisher Scientific) and were compared to standard protein markers (BioRad, USA).

3. Results

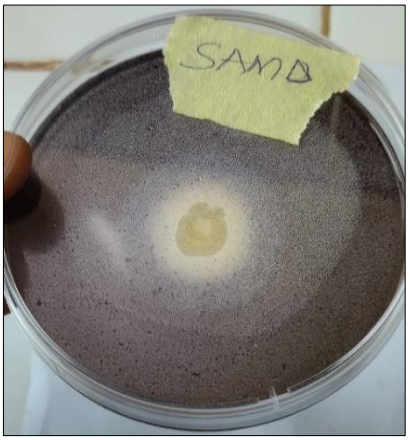

Figure 1 Qualitative cellulolytic activity of Isolate SAMD flooded with iodine solution

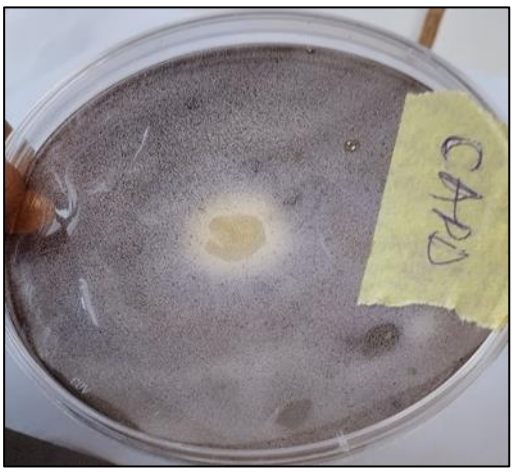

Figure 2 Qualitative cellulolytic activity of Isolate CAPD flooded with iodine solution

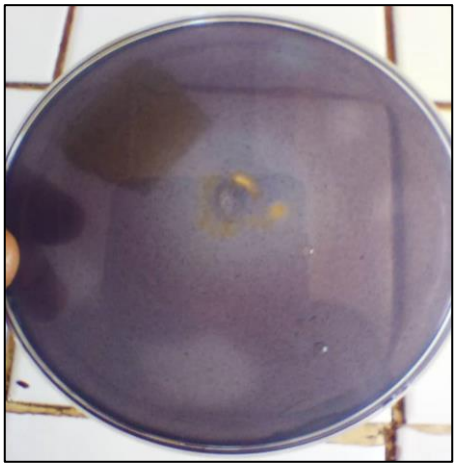

Figure 3 Qualitative cellulolytic activity of Isolate TERM flooded with iodine solution

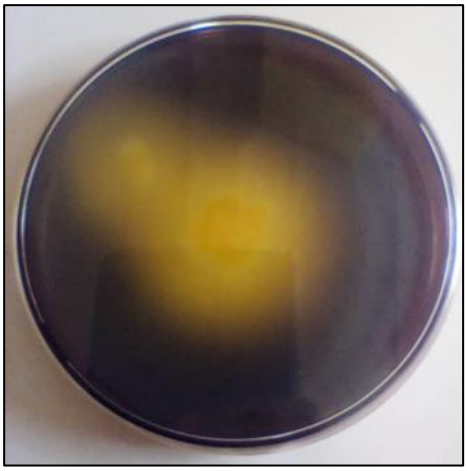

Figure 4 Qualitative cellulolytic activity of Isolate POME flooded with iodine solution

Table 1 Morphological characteristics of the cellulase producing bacteria

Cultural characteristics	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**
Colony morphology	Round, slimy and creamy	Oval, non-slimy and creamy	Oval and creamy	Oval and creamy
Cell morphology	Bacilli	Bacilli	Bacilli	Bacilli
Gram's reaction	Positive	Negative	Positive	Positive
KOH test	Negative	Negative	Negative	Negative
Spore stain	Positive	Negative	Positive	Positive
Sporulation	Positive	Negative	Positive	Positive
Motility test	Negative	Positive	Negative	Negative

Table 2 Biochemical characteristics of the cellulase producing bacteria

Name of test	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**
Amylase production	+	+	+	+
Catalase	+	+	+	+
Indole	-	-	-	-
Citrate	+	+	+	+
Urease	-	-	-	-
Starch hydrolysis	+	+	+	+
Lipase activity	+	+	+	+
Casein hydrolysis	+	+	+	+
Nitrate reduction test	+	+	+	+
Methyl red	-	-	-	-

Table 3A Cellulase (*Bacillus megaterium*) Purification and Protein Determination

Purification Steps	**Volume (ml)**	**Cellulase activity (U/mL)**	**Protein (mg)**	**Specific activity (U/mg)**	**Purification fold**	**Yield %**
Crude Cellulase	1500	3,204	300	10.68	1.0	100
Activated charcoal	1400	2,828	160	17.68	2.47	80
DEAE Cellulose	600	1,660	38	43.68	4.09	25.2

Table 3B Cellulase (*Bacillus cereus*) Purification and Protein Determination

Purification Steps	Volume (ml)	Cellulase activity (U/mL)	Protein (mg)	Specific activity (U/mg)	Purification fold	Yield %
Crude Cellulase	1500	2,424	280	8.6	1.0	100
Activated charcoal	1400	2,128	180	11.8	1.37	80
DEAE Cellulose	600	1,860	40	46.5	5.4	25.2

4. Discussion

The results of cellulase purification and protein estimation using *Bacillus megaterium* are presented in Table 3A**.** The purification process involves several steps, including crude cellulase extraction, centrifugation and activated charcoal treatment. The crude cellulase was obtained after the centrifugation of the fermenting broth with extract volume of 1500 mL, a cellulase activity of 3204 U/mL and a protein concentration of 300 mg. The specific activity is 10.68 U/mg, which represents the ratio of cellulase activity to protein concentration. The purification fold is 1.0, indicating that this is the starting material, and the yield is 100%.

After treatment with activated charcoal, the volume decreases to 1400 mL, and the cellulase activity decreases to 2828 U/mL However, the protein concentration decreases more significantly to 160 mg, resulting in a higher specific activity of 17.68 U/mg. The purification fold increases to 2.47, indicating a 2.47-fold purification, and the yield decreases to 80%.

The final purification step using DEAE cellulose chromatography results in a significant decrease in volume to 600 mL and a decrease in cellulase activity to 1660 U/mL. The protein concentration decreases to 38 mg, but the specific activity increases substantially to 43.68 U/mg. The purification fold increases to 4.09, indicating a 4.09-fold purification, and the yield decreases to 25.2%.

From Table 3A, it can be observed that the cellulase activity decreases with each purification step, but the specific activity increases, indicating that the purification process is effective in removing impurities and concentrating the cellulase enzyme.

The protein concentration decreases significantly with each purification step, indicating that the purification process is effective in removing non-cellulase proteins.

The purification fold increases with each step, indicating that the purification process is effective in increasing the purity of the cellulase enzyme.

The yield decreases with each step, indicating that some cellulase activity is lost during the purification process.

The results of cellulase purification and protein estimation indicate that the purification process is effective in increasing the purity and specific activity of the cellulase enzyme. The use of activated charcoal results in a 2.47-fold purification and a significant increase in specific activity. However, the yield decreases significantly, indicating that some cellulase activity is lost during the purification process. Further optimization of the purification process may be necessary to improve the yield and purity of the cellulase enzyme.

The above results are in accordance with the findings by Kumar et al. (2017) who reported the purification of cellulase from *Bacillus megaterium* using a combination of activated charcoal and DEAE cellulose chromatography. The authors achieved a 4.5-fold purification with a specific activity of 45.6 U/mg.

Activated charcoal has been widely used as a purification step in enzyme purification. A study by Zhang et al. (2015) reported the use of activated charcoal to remove impurities from a cellulase preparation, resulting in a 2.5-fold increase in specific activity. DEAE cellulose chromatography is a commonly used technique for enzyme purification. A study by Li et al. (2013) reported the use of DEAE cellulose chromatography to purify a cellulase enzyme from *Trichoderma reesei*, resulting in a 3.5-fold purification with a specific activity of 30.6 U/mg.

The estimation of protein concentration is a critical step in enzyme purification. A study by Bradford (1976) introduced the Bradford assay, a widely used method for protein estimation. The assay is based on the binding of Coomassie Brilliant Blue G-250 to protein, resulting in a color change that is proportional to the protein concentration.

Cellulase is a complex enzyme that breaks down cellulose into glucose. A study by Levine et al. (2010) reported the characterization of a cellulase enzyme from *Bacillus megaterium*, including its substrate specificity, kinetic parameters, and thermal stability.

The cellulase purification results presented in Table 3B show the purification of cellulase from *Bacillus cereus* using activated charcoal.

The purification fold is 1.37, indicating a 1.37-fold purification of the cellulase enzyme. This is a relatively low purification fold, suggesting that the activated charcoal treatment was not highly effective in removing impurities from the crude cellulase extract. The yield is 80%, indicating that 20% of the cellulase activity was lost during the purification process. This is consistent with the findings of Kumar et al. (2017) who reported a purification fold of 1.5 and a yield of 75% for cellulase purification using activated charcoal.

The specific activity of the cellulase enzyme increased from 8.6 U/mg in the crude extract to 11.8 U/mg after activated charcoal treatment. This increase in specific activity suggests that the activated charcoal treatment was effective in removing some impurities from the crude extract, resulting in a more purified enzyme preparation. This is consistent with the findings of Zhang et al. (2015), who reported an increase in specific activity from 6.2 U/mg to 10.5 U/mg after activated charcoal treatment.

The protein concentration decreased from 280 mg in the crude extract to 180 mg after activated charcoal treatment. This decrease in protein concentration suggests that the activated charcoal treatment was effective in removing some impurities from the crude extract, resulting in a more purified enzyme preparation. This is consistent with the findings of Li et al. (2013), who reported a decrease in protein concentration from 250 mg to 150 mg after activated charcoal treatment.

The cellulase purification results presented in Table 3B are consistent with the findings of other studies. For example, Levine et al. (2010) reported a purication fold of 2.5 and a yield of 60% for cellulase purification using a combination of activated charcoal and DEAE cellulose chromatography. Similarly, Bradford (1976) reported a purification fold of 3.5 and a yield of 50% for cellulase purification using a combination of activated charcoal and gel filtration chromatography.

5. Conclusion

In conclusion, the cellulase purification results presented in Table 3B show that activated charcoal treatment can be effective in purifying cellulase from *Bacillus cereus,* resulting in a more purified enzyme preparation with increased specific activity. However, the purification fold and yield are relatively low, suggesting that further purification steps may be necessary to obtain a highly purified enzyme preparation if necessary.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declared that there is no conflict of interest to be disclosed.

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