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Optimization of Cellulase Enzyme Produced by *Pseudomonas aeruginosa* and its Potentiality to Remediate Health Hazard Pollutants from the Environment

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Abstract

Microbial cellulases have a variety of applications including agricultural, industrial, pharmaceutical, medical, and pollution research. In this research work, *Pseudomonas aeruginosa* was isolated, screened, and employed for optimization studies for the production of cellulase. At the initial stage, CMCase production by the bacterial isolate in selected Winstead's broth was found 84.93 UmL^{-1} and then sequentially improved up to 253.85 UmL^{-1} after incubation at 40˚C, pH 7.5 for 3 days. Sawdust and peptone were noted as the most potent carbon and nitrogen ingredients for the induction of cellulase production by the respective isolate. The degradation activity of the crude enzyme was also boosted up to 25-fold when several factors during the enzyme-substrate reaction phase were optimized. The results demonstrate that *Pseudomonas aeruginosa* has the potential to produce alkaline cellulase and utilize sawdust, a health hazards pollutant as the best substrate in the production medium.

Keywords: Cellulase, *Pseudomonas aeruginosa*, Pollutant, Remediation.

Introduction

Cellulose, a polysaccharide fluctuates in different natural substances by chain length[1]. Cellulase is a group of enzymes that is mainly composed of at least three enzymes endoglucanases, exoglucanases, and β-glucosidase[2,3]. These enzymes are capable of hydrolysis of cellulose to β-D-glucose[4].

Lignocellulosic pollutants such as sawdust have appeared as one of the most frequent inimical agents toward the environment and human health (e.g., inhibition of respiration and carcinogenic effects). Sawdust is a light brown or tan fibrous powder-like substance produced when timber is processed[5]. Sawdust particles can occur over a wide range of particle sizes during wood processing. According to the definition of Aerodynamic Equivalent Diameter (AED) of a particle, the inhalable fraction (< 100 μm AED) can be breathed into nose or mouth, the thoracic fraction (< 25 μm AED) can penetrate head airways and enter lung airways and the respirable fraction (< 10 μm AED) can penetrate beyond terminal bronchioles to gas exchange region[6,7]. Therefore, when the dust particle sizes are small, they become airborne and pose more serious issues. Burning of sawdust produces health hazards and toxic gasses such as carbon monoxide, nitrogen dioxide, and sulfur dioxide. Inhaling sawdust into the lungs also can cause lung diseases[8]. In various ecosystems, microbial degradation of cellulosic substances has been observed at an outstanding frequency which offers a way for remediation of polluted environments. Natural cellulolytic microbes express greater efficacy (in case of cellulose degradation) than laboratory-cultured microorganisms in usual cases[9]. Both aerobic and anaerobic bacteria such as *Acinetobacter junii, Acetivibrio cellulolyticus, Bacillus subtilis, Butyrivibrio fibrisolvens, Cellulomonas biazotea, Clostridium thermocellum, Pseudomonas* sp. are capable of the degradation of cellulosic substances[10,11].

Cellulase enzymes have diverse industrial uses in beverage, textile, fuel, food, and remarkably in bio-ethanol production. From an industrial point of view,

crucial challenges are expensive production and low output of desired enzymes[12-17]. Bacterial enzyme production is substantially affected by nutritional ingredients and some physical factors including incubation time, hydrogen ion concentration, aeration, and temperature[18-20]. From a fermentation perspective, optimization of several physicochemical parameters like pH, temperature, incubation time, ionic concentration, nutrient elements, etc. has a fundamental impact on microbial growth and metabolic activities[21-23].

Employment of cheaper carbon and nitrogen sources in production media can efficiently nourish cellulolytic microbes and thus minimize production costs[24]. The present study focused on the isolation of naturally occurring cellulolytic bacteria, selection according to their potency, identification of the selected isolate, and augmentation of cellulase production with activity.

Materials and Methods

Sampling and isolation of cellulose-degrading bacteria

Sampling was accomplished from soil sites (Forest of University of Chittagong and different sawmills located in Chattogram district, Bangladesh). Isolation procedure was preceded by plating serially diluted soil suspensions on enrichment media (Carboxymethyl cellulose agar media containing peptone-5gm, beef extract-3gm, agar-15gm, CMC-2.5gm, and distilled water-1000 ml). After incubation, Gram's iodine solution was utilized as an inundating agent in screening procedure[25]. Formation of clearing zones by hydrolysis of CMC indicated the capability of selected bacteria for producing cellulases. The isolates were further screened for their cellulolytic ability in liquid Winstead's broth (Asparagine - 2 gm, CMC- 10 gm, Tween 80- 1 ml, $MgSO_4$.$7H_2O$- 2.5 gm, K_2HPO_4- 3 gm, Distilled water- up to 1000 ml) at pH 7.0 and incubated at 37°C for 48 hours in an orbital shaker incubator at 150 rpm. Consequently, centrifugation method was proceeded at 10,000 rpm for 25 min at 4°C for collection of supernatants[26].

Enzyme Assay

In case of CMCase assay, Nelson's modification of Somogyi method was applied to quantify the concentration of liberated reducing sugar[27]. Keeping pace with international standards, activity of the enzyme was expressed by the quantity of glucose liberated by the action of crude cellulase (gm/ml/hr)[28]. In the course of enzyme assay, 2 ml supernatant and 2 ml of 1% CMC (citrate-phosphate buffer was utilized for preparation) combination were applied. Then 1ml of corresponding citrate-phosphate buffer was incorporated into this mixture. Thermal instrument like a water bath providing a holding temperature of 37˚C for 2 hours was implemented to create a suitable environment for proper mixing between enzyme and substrate. Then 1 ml of enzyme-treated sample was withdrawn and transferred into a test tube. One ml of freshly prepared alkaline copper reagent was added to it. The respective mixture was stewed in a boiling water bath for 20 minutes. Running water was used to decrease temperature, then 1 ml of Arsenomolybdate color reagent was incorporated into this tube, vortexed carefully and diluted up to 25 ml with distilled water. Consecutively, absorbance was taken at 500 nm in a Spectrophotometer (T60 UV-VIS, PG instrument Ltd, UK). Comparison between absorbance of subject mixture and absorbance of 'D-glucose' (25-200 µg) (plotted in standard curve) was executed to calculate the amount of liberated reducing sugar. Boiled enzymes and uninoculated media were treated as control tube.

Optimization of cellulase production

Microorganisms produce bulk enzymes at highest level at optimized cultural conditions. Winstead's broth was employed for the optimization of different factors of cultural conditions. For enzyme assay, cells were separated as pellets from culture supernatant by centrifugation at 10,000 rpm, for 25 minutes, at 4°C.

Effect of temperature, hydrogen ion concentration and incubation period

To ascertain the appropriate conditions for enzyme production at paramount level, the subject isolate was incorporated into the production media with distinct pH ranging from 5.0 to 8.0 with incubation at 27, 37, 40 and 45℃ and for 2, 3 and 4 days and their effect on enzyme production was evaluated.

Effect of carbon ingredients in production media

Different carbon sources (CMC, cellobiose, avicel, salicin, sawdust, leaf, and rice straw) were used individually to observe their effect on the rate of cellulase production. The bacterium was grown into the medium (at optimized pH 7.5) supplemented with carbon source and incubated at an optimized temperature (40℃) for 3 days.

Effect of nitrogen ingredients in production media

To enhance enzyme production rate, the effect of different nitrogen sources (asparagine, peptone, yeast extract, KNO_3, and NH_4Cl) was investigated by inoculation of the selected bacterium into the media supplement with an individual nitrogen source and incubated at optimized cultural conditions.

Factors affecting enzyme (crude) activity

Enzyme activity depends on a number of physicochemical parameters. The optimum level of these physicochemical parameters was determined by studying the enzyme-substrate reaction phase in varying conditions.

Effect of temperature

Five discrete temperatures (27, 37, 40, 45, and 50℃) were examined to detect the influence of temperature on cellulase activity.

Effect of hydrogen ion concentration

The enzyme-substrate mixture was incubated at various pH (5.0-9.0) at optimum temperature followed by assaying the enzyme activity to determine the influence of pH on cellulase activity.

Effect of substrate concentration

To determine the saturation point of enzyme-substrate reaction mixture, different concentrations of substrate (0.5, 1, 1.5, 2, and 2.5%) were examined at optimum temperature and pH.

Effect of incubation time

The reaction between enzyme and substrate was conducted at different incubation periods (10-160 mins) with optimized pH, temperature, and substrate concentration.

Identification of the selected isolates

Different morphological, cultural, and biochemical characteristics were determined to identify the selected bacterial isolate. As per guideline of Bergey's Manual of Determinative Bacteriology, cultural, morphological, and biochemical features were considered for the identification of the respective bacteria[29].

Results and Discussion

Isolation and selection of cellulolytic bacteria

Primarily, 16 bacterial colonies were isolated by using an enrichment media from the soil of forest and sawmill. Cellulose (CMC) hydrolysis method was employed for screening isolates (by cellulase activity of the concerned isolates). Among the bacterial isolates, one isolate showed potent cellulolytic activity which was selected for detailed study (Figure 1).

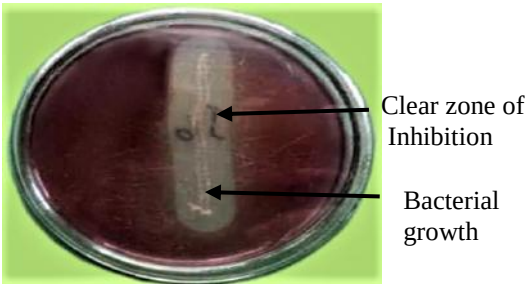

Figure 1. Primary screening for cellulolytic activity by the selected bacteria on Nutrient agar medium containing CMC.

Natural environments like gut of termites or grass carp, soil, mangrove soil, woody organisms (caterpillar, bookworm, snail) and kitchen wastes have been proved

to be genuine source of potential cellulolytic bacteria in previous studies[30-37].

Optimization of cultural conditions

Industrial enzyme production is very expensive which demands optimization of conditions of bacterial culture from an economic perspective. Physicochemical parameters which have remarkable impact on cellulose degradation were studied. During large-scale enzyme production, influencing parameters responsible for the elevation of enzyme production demands a clear understanding and absolute control[38]. Optimization of cultural conditions was investigated to obtain the highest growth and maximum rate of cellulase production (Figure 2a-2c).

Effect of temperature

Bacteria inoculated into the culture medium and incubated at various incubating temperatures ranging from 27 to 45°C to perceive the effect of temperature on cellulase production. The bacterial isolate exhibited maximum CMCase production (101.92 UmL^{-1}) at 40°C (Figure 2a). Further increase in temperature, the rate of enzyme production gradually lost. Immanuel *et al.*[39] reported the highest endoglucanase activity at 40°C from *Cellulomonas*, *Bacillus*, and *Micrococcus*. Fagade and Bamigboye[40] also noted maximum cellulase activity at 40°C from *B. licheniformis* I and II; Lin *et al.*[41] for *B. thuringiensis* and Alam *et al.*[42] for *Streptomyces omiyaensis.*

Effect of pH

The isolate showed a steady increase of CMCase production over a wide range of pH (5.0– 8.0). It was noted that the rate of enzyme production increased from pH 5.0 in the production medium until enzyme production found maximum (119.87 UmL^{-1}) at pH 7.5 (Figure 2b), followed by gradual decline. Cellulase production was found maximum between pH 7.0 and 9.0 by others reports[33,43-46].

Effect of incubation period

Incubation period is another significant influencing parameter from enzyme production perspective. To determine an optimal incubation period, the CMCase production by the subject isolate was measured at various incubation period (Fig. 2). In our study, the maximum rate of CMCase production was found within 3 days of incubation (126.28 UmL^{-1}) (Figure 2c). Increase in incubation time over 3 days revealed slow decline in enzyme production. This negative induction of cellulase production with prolonged incubation time might be the result of the scarcity of nutrients in the production medium or agglomeration of unfavorable byproducts in the fermentation media[47]. Moreover, Melo *et al.*[48] opined that reduced water activity, and denaturation of enzymes are responsible for decline in enzyme production with extended incubation time. The basis of their opinion was fluctuated in pH in the course of the fermentation process. Furthermore, Singh *et al.*[49] noticed that enhancing cellobiose effects can also decline cellulase production when incubation time extends.

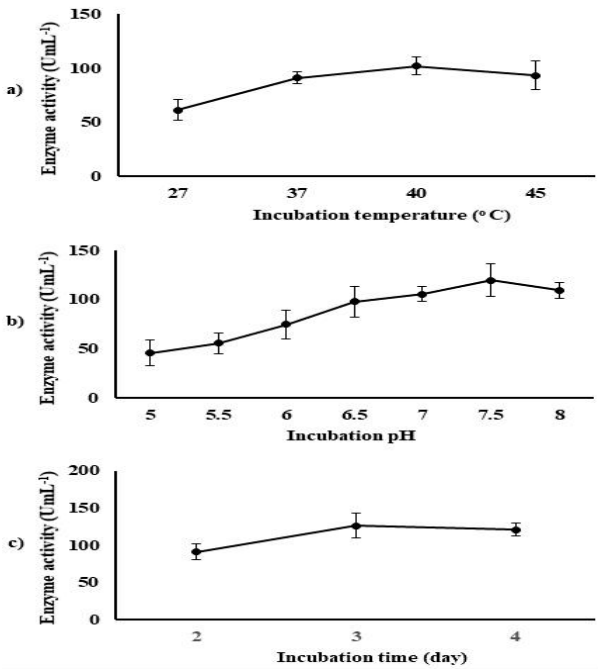

Figure 2. Effects of influencing factors on cellulase production of *Pseudomonas aeruginosa*. The bacteria were cultured under different incubation temperatures (a), initial pH (b), and incubation period (c). Cellulase production was assayed in triplicates (n = 3). The vertical bars represent the standard error of mean.

Effect of carbon ingredient

To evaluate the impact of carbon sources on cellulase production rate by selected bacterial isolate, 7 different carbon sources were examined (Figure 3).

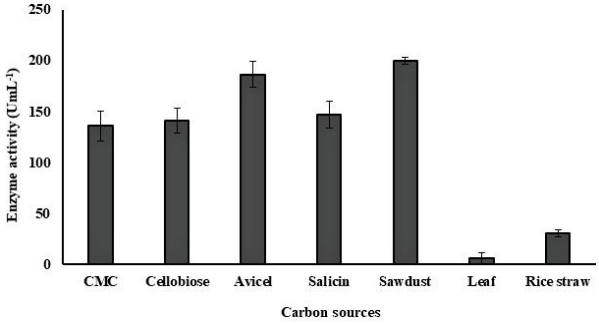

Figure 3. Effect of natural and artificial carbon ingredients on cellulase production of selected isolate bacteria *Pseudomonas aeruginosa*. CMCase activity was assayed in triplicates (n = 3). The vertical bars represent the standard error of mean.

The effect of carbon sources on the production of cellulose results exhibited that the isolate *Pseudomonas aeruginosa* could employ several carbon ingredients and cellulase production reached its peak when sawdust was incorporated into the medium (200 UmL^{-1}) (Figure 3). Other reports showed that commercially available carbon sources such as CMC can also be a notable option for production of cellulase at supreme level. Sawdust burning releases different health hazards and toxic gasses. Inhaling sawdust into the lungs also can cause lung diseases. Therefore, the research findings indicated the potentiality of the isolate to remediation of health hazards wood dust from the environment[50-52].

Effect of nitrogen ingredient

Organic and inorganic nitrogen sources were utilized under optimum condition to assess their induction propensity for cellulase production (Figure 4). The result indicated that the rate of enzyme production varied remarkably on the constituents of the production media and the highest enzyme production was obtained in case of peptone when it was employed as nitrogen ingredients (253.85 UmL^{-1}) (Figure 4). According to previous investigations, nitrogen sources of organic origin were more capable of enzyme induction than inorganic nitrogen sources[44,53]. Therefore, the maximum induction of cellulase production by *Pseudomonas aeruginosa* was observed in the presence of sawdust and peptone as nutritional ingredients (Figure 4).

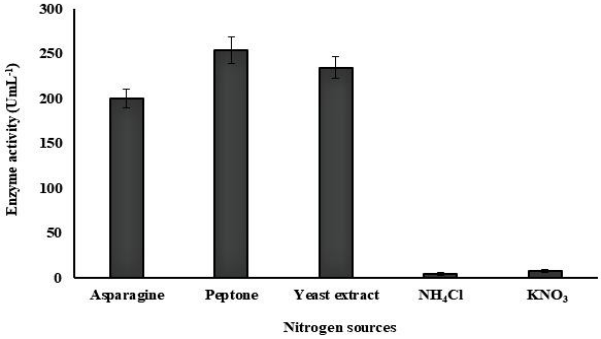

Figure 4. Effect of several organic and inorganic nitrogen ingredients on cellulase production. CMCase activity was assayed in triplicates (n = 3). The vertical bars represent the standard error of mean.

Optimization of different factors affecting enzyme (crude) activity

An attempt was made to optimize different factors for getting maximum enzyme activity. Effect of temperature on cellulase efficiency was explored by providing the enzyme-substrate reaction mixture at distinct temperatures ranging from 27 to 50℃ and found highest enzyme activity at 40℃ temperature (553.85 UmL^{-1}). But above 40℃ the activity of enzymes decreased drastically (Figure 5a).

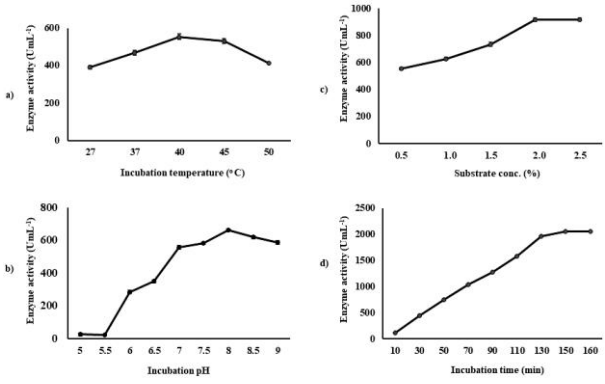

Figure 5. Effects of different factors involved in cellulase activity of *Pseudomonas aeruginosa*. The enzyme substrate reaction was carried out under different temperatures (a), pH (b), substrate concentration (c), and reaction times (d). Cellulase activity was assayed in triplicates (n = 3). The vertical bars represent the standard error of mean.

Table 1. Consecutive stages of experiments and attained augmentation in cellulolytic potential of strain *Pseudomonas aeruginosa*.

Stages	Constant parameter	Variable parameters	Optimized findings of parameters	Cellulase activity (UmL^{-1})
Optimization of cultural conditions:				
Level 1	Basal medium + Incubation time + pH + Temperature	No variable parameter	-	84.93
Level 2	Basal medium + Incubation time + pH	Temperature	40˚C	101.92
Level 3	Basal medium + Optimum incubation time + Optimum temperature	pH	7.5	119.87
Level 4	Basal medium + Optimum temperature + pH	Incubation time	3days	126.28
Level 5	Basal medium + Optimum incubation time + Optimum temperature + Optimum pH	Carbon sources	Sawdust	200.00
Level 6	Basal medium + Optimum incubation time + Optimum temperature + Optimum pH + Sawdust	Nitrogen sources	Peptone	253.85
Optimization of enzyme-substrate reaction phase:				
Level 7	Crude enzyme + pH + reaction time	Temperature	40˚C	553.85
Level 8	Crude enzyme + Optimum ES temperature+ Reaction time	pH	8	662.82
Level 9	Crude enzyme + Optimum ES temperature+ Optimum ES pH+ Reaction time	Substrate concentration	2%	919.23
Level 10	Crude enzyme + Optimum ES temperature + Optimum ES pH	Reaction time	150 min	2055.77

Note- ES=Enzyme-Substrate

Highest cellulase activity was exerted at pH 8.0 (662.82 UmL^{-1}) and 40℃ by the isolate *Pseudomonas aeruginosa*. An exponential state of enzymatic activity was described between pH 5.5 to 8.0, and then declined (Figure 5b). Optimum substrate concentration was found at 2% (919.23 UmL^{-1}) (Figure 5c). Enzyme-substrate reaction mixture was also carried out in different time intervals (10 to 160 minutes) to find out maximum activity. It was observed that the highest enzyme activity was achieved after 150 min of enzyme-substrate reaction (2055.77 UmL^{-1}) (Figure 5d). Findings remarked in our investigation concurrence

with the research of some workers[54,55]. Optimal temperature 37°C and pH 7.0 was found for maximum CMCase activity according to the report of Khatiwada *et al*.[56] On the other hand, Islam *et al*.[57] observed optimum cellulase activity at slightly acidic pH 5.5 and moderately high temperature 50°C for incubation time of 50 mins. Degradation of cellulosic substances into simple carbohydrates by microorganisms has been found for a long time. However, in view of industrial and environmental perspectives, there is also a necessity to hunt more potential cellulose degraders which signifies this study.

Overview of enhanced cellulolytic potential

The summary of acquired magnification in cellulolytic potential of *Pseudomonas aeruginosa* is summarized in (Table 1). After optimization of culture conditions, the enhancement of cellulase activity was found about 3fold. On the other hand, after optimization of the enzyme-substrate reaction, the activity of crude enzyme increased by about 25fold.

Identification of bacteria

The selected isolate was identified as *Pseudomonas aeruginosa* following Bergey's Manual of Determinative Bacteriology. Characterized features of these potent cellulolytic bacteria were scrutinized for identification purposes. Ideal features of bacteria retrieved from Bergey's Manual of Determinative Bacteriology were considered as a standard in this regard[29]. Ultimately, the studied potential bacterium was identified as *Pseudomonas aeruginosa*.

Conclusions

Accumulation of agricultural, industrial, municipal, and anthropogenic waste creates a serious threat to the environmental components. Utilization of lignocellulosic waste by microbial biodegradation can effectively reduce pollution to a great extent. Moreover, cellulase enzymes and end products of lignocellulosic substrates have a variety of applications including industry, pharmaceuticals, and medicinal uses as well as research etc[58]. In the present investigation, *Pseudomonas aeruginosa* showed maximum cellulolytic activity in alkaline conditions by utilizing sawdust, making it a potential candidate for remediation of health hazard pollutants from the environment and promoting sustainable environmental management. So, the conclusion derived from the present study is that the bacterium *Pseudomonas aeruginosa* has the potential to produce alkaline cellulases in optimized conditions and can also be applied to reduce environmental pollution and promote sustainable environmental management.

Declarations

It is certified that the study was carried out by the authors and the content of this manuscript was not published in any journal.

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Competing Interests

The authors proclaim that they do not have any known competing financial benefits or individual relationships that could have seemed to affect the work mentioned in this paper.

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