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Isolation of cellulolytic bacteria and production of cellulase using different substrates

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Abstract

Background and aim: Cellulase is an enzyme system that catalyzes the hydrolysis of cellulose into reducing sugars. Cellulases are important industrial enzymes and have wide range of applications in industries such as food, brewery, wine, paper and pulp, textile, feed, detergent, in agriculture and in the production of bioethanol. **Methodology:** In this work, bacteria were isolated from cow dung and screened for the production of cellulase. **Results:** Highest enzyme production was shown by the isolate 10. Based on morphological and biochemical reactions, the isolate was identified as *Bacillus sp.* Optimum pH and temperature for the growth of the isolate was found to be 8.0 and 40°C respectively. Different agro- based wastes such as paddy straw, wheat bran; tamarind seed powder and coconut shell powder were used as substrates for the production of cellulase by solid state fermentation. The novel substrates- tamarind seed powder and coconut shell powder were found to be promising substrates for the production of cellulase. Process optimization was done for different substrate concentrations, pH and temperatures. Maximal enzyme activity with paddy straw and wheat bran was found at 20% substrate concentration, pH 7.0 and 40°C; with tamarind seed powder, at 25% substrate concentration, pH 8.0 and 40°C; and with coconut shell powder, at 20% substrate concentration, pH 9.0 and 50°C. Partial purification of the enzyme was carried out by ammonium sulfate precipitation followed by dialysis. The activity of the precipitated enzyme was determined by gel diffusion method and maximum enzyme activity was recorded at 40% ammonium sulfate concentration.

***Keywords**:* Cellulase; Cow dung; *Bacillus* sp.; Solid state fermentation; Optimization; Partial purification

1. Introduction

Cellulose, being a major component of the plant cell wall, is the most abundant biopolymer on earth. Cellulose is a linear chain of D- glucose units linked together by β-1, 4- glycosidic bond[1]. The β - 1, 4- glycosidic linkages in the cellulose can be hydrolyzed by cellulolytic enzyme, cellulase. The cellulase is a group of enzymes which is composed of at least three different enzymes. Endoglucanase (endo-1,4- β-D-glucanase, EC 3.2.1.4) attacks randomly the internal linkages within the cellulose chain, creating free chain ends; exocellulase (exo-1,4- ß-D-glucanase, EC 3.2.1..91) hydrolyzes cellulose from the free ends creating mainly cellobiose as an end product, and β - glucosidase (EC 3. 2.1.21) hydrolyzes the cellobiose to glucose[2]. Different bacteria and fungi have been used for cellulase production[3,4]. Cellulolytic properties of certain bacterial species such as *Pseudomonas sp., Cellulomonas sp., Cellovibrio sp., and Sporophytophaga sp.* have been reported[5]. Cellulases have a wide range of industrial applications. The major industrial applications of cellulases are in textile industry for 'biopolishing' of fabrics and producing stonewashed look of denims, in household laundry detergents for improving fabric softness and brightness[6], in food, leather, paper/ pulp industries and also used in the fermentation of biomass for the biofuel production. Besides, cellulases are also used in ruminant nutrition for improving digestibility, in fruit processing and another

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emerging application is deinking of paper[7].

Solid state fermentation (SSF) is the growth of organisms on moist substrates in the absence of free- flowing water. The use of SSF for production of enzymes and other products has many advantages over submerged fermentation. SSF permits the use of agricultural and agro- industrial residues as substrates for the production of product with high commercial value[8, 9]. Utilization of agro- based waste helps solving pollution problems[10]. There are few reports of bacterial strains being used successfully for the production of enzymes by using SSF[11-14].

The aim of this study was to isolate and identify cellulolytic bacteria from cow dung and to optimize different parameters for the production of cellulases using different agro- based substrates such as paddy straw, wheat bran, tamarind seed powder and coconut shell powder; and compare the yields of enzyme in different substrates.

2. Materials and methods

2.1 Isolation of cellulase producing bacteria

Cow dung sample was collected from a local farm. 1g of sample was serially diluted in sterile distilled water up to 10^{-6} dilutions. 1ml of each dilution were poured into agar plates and incubated for 24hrs at 37°C.

2.2 Primary screening for cellulase activity and maintenance of pure culture

The bacterial isolates were screened for the production of cellulase by streaking them individually on Carboxy Methyl Cellulose (CMC) agar containing (g/l) KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.01; NH_4NO_3, 0.3; CMC, 10.0 and agar, 12.0. The pH was adjusted to 7.0[15].The plates were incubated for 24 hours at 37°C. The plates were flooded with 1% (w/v) Congo red solution for 15 min followed by 1M NaCl for 15 min. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select the highest cellulase producer. The largest ratio was assumed to contain the highest activity[16].

The best producer bacteria were plated on nutrient agar plates and incubated for 24h at 37°C and stored at 4°C.

2.3 Identification of cellulase producing bacteria

The best producer bacteria was identified by means of morphological and biochemical characterizatics. The morphological characteristics were done by gram staining and endospore staining. Motility test was also performed.

Biochemical tests performed included Catalase test, Oxidase test, Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Starch hydrolysis, Gelatin liquefaction, Nitrate reduction, and Carbohydrate fermentation test.

2.4 Study of growth characteristics- pH and temperature

The optimum pH and temperature for the growth of the bacterial isolate was determined by Turbidometry method. Growth at different pH (5, 5.5, 6, 6.5, 7, 7.5 and 8) and temperature (10°C, 20°C, 30°C, 37°C, 40°C and 50°C) were investigated. All investigations were carried out using nutrient broth. The absorbance of the broths after incubation was measured using spectrophotometer at 540 nm.

2.5 Collection and processing of substrates

The agro- based wastes- wheat bran, paddy straw, and tamarind seed powder were collected from a local market and coconut shells were collected from households.

Lignocellulosic substrates- paddy straw and coconut shell were subjected to pretreatment so as to make cellulose readily available for degradation by bacteria.

Paddy straw was washed thoroughly with water and dried. Dried paddy straw and coconut shells were powdered using pulverizer. Powdered paddy straw and coconut shell were soaked in 1% sodium hydroxide in 1:10 (substrate: solution) ratio for 2h at room temperature, washed and autoclaved at 121°C for 1h[17,18]. Treated substrates were then filtered and washed with distilled water until the wash water becomes neutral.

2.6 Solid State Fermentation

Fermentation media were prepared by adding 50ml of basal Mineral Salt Medium (MSM)[19] to 10g of each of the substrates in separate 250ml Erlenmeyer flasks, followed by autoclaving at 121°C for 15min. 1ml of 24h old broth culture of the bacteria was inoculated into each of the flasks containing fermentation media. The flasks were incubated at 37°C for 48h in a shaker at 120- 150 rpm.

2.7 Preparation of crude enzyme

Crude enzyme was extracted using phosphate buffer (0.1M, pH 7.0) at 1:5 (w/v), with a contact time of 30 min and agitation at 150 rpm at room temperature on a rotary shaker. Dampened cheese cloth was used to filter the extract[20-23]. After extracting twice, the extracts were centrifuged at 10000 rpm at 4°C for 15min. The supernatant was used as the source of enzyme.

2.8 Cellulase assay

Cellulase activity was measured by DNS method[24] using CMC as substrate. The reaction mixture contained 0.5ml of 1% (w/v) CMC in 0.1M phosphate buffer (pH 7.0) and 0.5ml of culture supernatant. The mixture was incubated at 40°C for 30min. After incubation, the reaction was terminated by adding 1ml DNS and subsequently placing the tubes in a water bath at 100°C for 5min. to the warm

reaction mixture, 1ml of 40% Rochelle salt (sodium potassium tartarate) solution was added to stabilize the colour. The reaction mixture was cooled and the solution was made up to 10ml with distilled water. The absorbance was measured at 550nm using spectrophotometer. Control was prepared with enzyme boiled for 10min. The enzyme activity was determined by using glucose standard curve. One unit of cellulase activity was expressed as µg of glucose liberated per ml enzyme per min.

2.9 Optimization of cellulase production

Effect of various parameters like substrate concentration, pH, temperature and nitrogen source on cellulase production were investigated using the four different substrates- wheat bran, paddy straw, tamarind seed powder and coconut shell powder. Solid state fermentation by shake flask method was carried out for 48h at 37°C in each case.

2.9.1 Effect of substrate concentration

To determine the optimum substrate concentration, fermentation was carried out at various substrate concentrations- 5%, 10%, 15%, 20% and 25%.

2.9.2 Effect of pH

Fermentation was carried out at pH ranging from 5.0- 9.0 to study their effect on cellulase production.

2.9.3 Effect of temperature

To determine the effect of temperature, fermentation was carried out at various temperatures- 20°C, 30°C, 40°C, and 50°C.

2.9.4 Effect of nitrogen source

For determining the effect of nitrogen sources on the production of cellulase, peptone and ammonium sulfate were used as nitrogen sources at concentrations 0.5% and 1%.

2.10 Partial purification of enzyme

Enzyme in the cell- free supernatant was precipitated with different concentrations (20- 80%) of solid ammonium sulfate separately. The precipitates were collected by centrifugation and the enzyme pellets were dissolved in 50mM phosphate buffer (pH 7.0). The dissolved pellet was dialyzed against same buffer.

2.11 Gel diffusion assay for detection of cellulase activity of precipitated enzyme

Gel diffusion assay was carried out in order to determine the cellulase activity of the precipitated enzyme.

Enzyme precipitated using different concentrations of ammonium sulfate were placed in wells punched in separate CMC agar plates and the plates were incubated at 30°C for 24h. After incubation, the plates were flooded with Congo red solution for 15min followed by rinsing with 1M NaCl solution for 10- 15 min. Zone of hydrolysis around the wells indicate cellulase activity. The diameter of clear zone in each plate was measured to determine which of the precipitate shows highest cellulase activity.

3. Results

3.1 Isolation of cellulase producing bacteria

11 isolates were obtained from cow dung, which were screened for cellulase production.

3.2 Primary screening for cellulase activity

All the 11 isolates showed growth on CMC agar and demonstrated positive results with Congo red. The ratios of clear zone diameter to colony diameter of the different isolates are shown in Table I.

3.3 Identification of cellulase producing bacteria

The bacterial isolate appeared as white colonies on CMC agar and nutrient agar. Microscopic examination showed that the isolate was Gram positive with paracentral, ellipsoidal spores. The results of biochemical results are tabulated in Table II.

Table I: Zone of hydrolysis of various isolates

Isolate number	Clear zone diameter (cm)	Colony diameter (cm)	Clear zone diameter/ Colony diameter
1	2.3	0.73	3.15
2	1.3	0.73	1.78
3	1.3	0.36	3.61
4	2.43	1.23	1.97
5	2.0	0.96	2.08
6	1.93	1.06	1.82
7	1.4	0.66	2.12
8	1.36	0.86	1.58
9	2.23	1.23	1.81
10	2.1	0.53	4.52
11	2.26	0.5	3.9

Table II: Results of biochemical tests

Sl. No.	Biochemical test	Result
1	Catalase	+
2	Oxidase	+
3	Indole	-
4	Methyl red	-
5	Voges- Proskauer	+
6	Citrate utilization	+
7	Starch hydrolysis	+
8	Gelatin liquefaction	+
9	Nitrate reduction	+
10	Motility	+
11	Carbohydrate fermentation:	
	Glucose	+
	Sucrose	+
	Xylose	-

(-) Negative and (+) Positive

Table III: Optical densities corresponding to various pH

pH	Optical density at 540nm
5.0	0.43
5.5	0.43
6.0	0.45
6.5	0.47
7.0	0.47
7.5	0.49
8.0	0.50

Table IV: Optical densities corresponding to various temperature

Temperature	Optical density at 540nm
10°C	0.02
20°C	0.09
30°C	0.18
37°C	0.41
40°C	0.43
50°C	0.19

On the basis of morphological and biochemical characteristics, the bacterial isolate was identified as ***Bacillus sp.*** It belongs to the group 1 of *Bacilli* i.e. ***Bacillus cereus*** group which is characterized by Gram positive bacteria producing central or terminal, ellipsoidal or cylindrical spores that do not distend the sporangium.

3.4 Study of growth characteristics- pH and temperature

Highest growth of the bacterial isolate was found at 40°C and at pH 8.0. Optical densities corresponding to various pH and temperature are tabulated in Table III and Table IV respectively.

3.5 Optimization of cellulase production

3.5.1 Effect of substrate concentration

In case of wheat bran, paddy straw and untreatedcoconut shell, highest cellulase activity was found at concentration of 20%, in case of tamarind seed at 25%, and 10% in case of pretreated coconut shell. The effect of various concentrations of the substrates is illustrated in Figure I.

3.5.2 Effect of pH

Highest cellulase activity in wheat bran and paddy straw was found at pH 7.0, in tamarind seed at 8.0 and in untreated coconut shell at 9.0. Figure II illustrates the effect of pH on cellulase production.

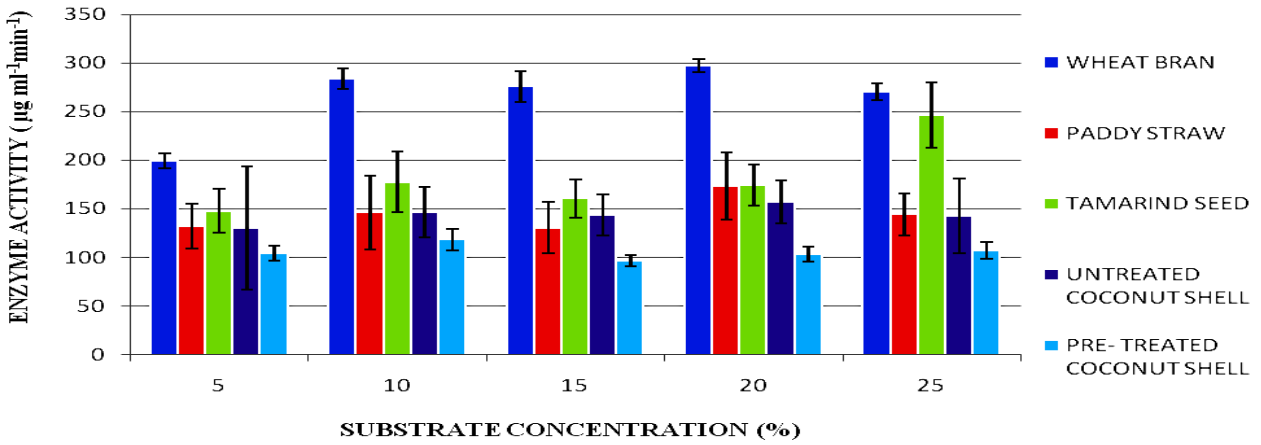

Figure I: Effect of substrate concentration on cellulase production

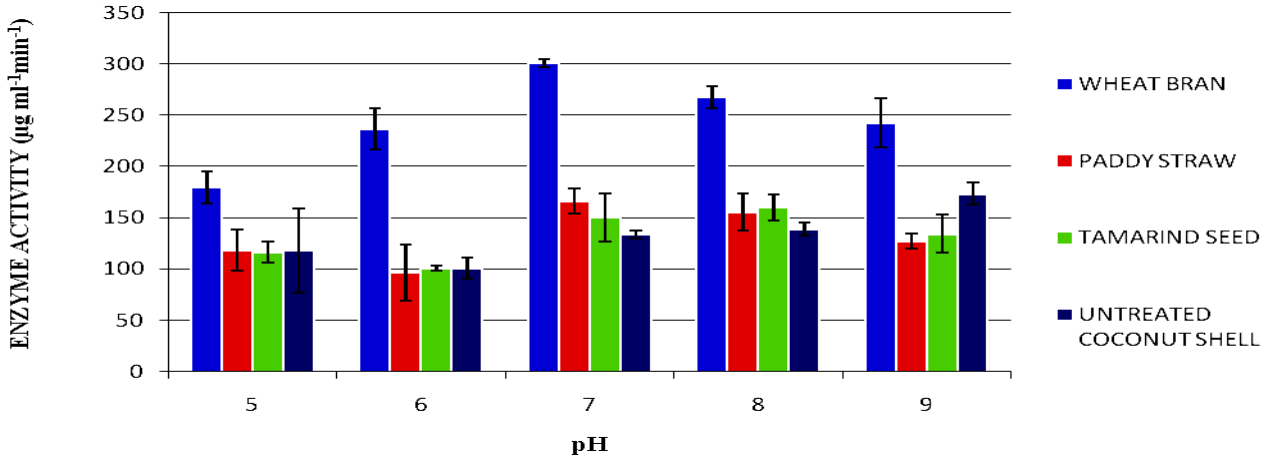

Figure II: Effect of pH on cellulase production

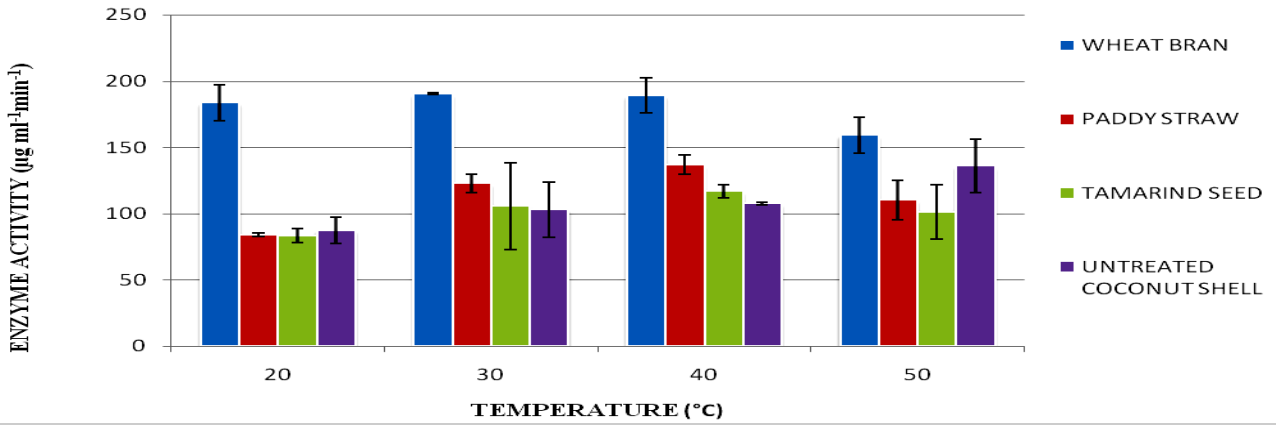

Figure III: Effect of temperature on cellulase production

3.5.3 Effect of temperature

With wheat bran, paddy straw and tamarind seed, highest cellulase activity was found at 40°C; and at 50°C with untreated coconut shell. The results are illustrated in Figure III.

3.5.4 Effect of nitrogen source

When peptone was used as nitrogen source, higher cellulase activity was observed at 0.5% peptone concentration with paddy straw, tamarind seed and untreated coconut shell, and at 1% peptone concentration with wheat bran. The results are illustrated in Figure IV.

When ammonium sulfate was used as nitrogen source, maximum activity was observed at 1% ammonium sulfate concentration in case of all the substrates. The results are illustrated in Figure V.

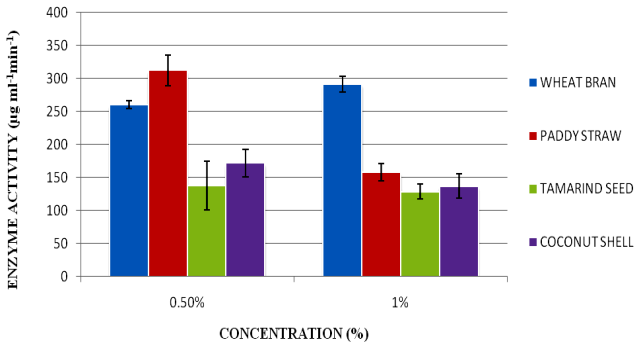

Figure IV: Effect of peptone concentration on cellulase production

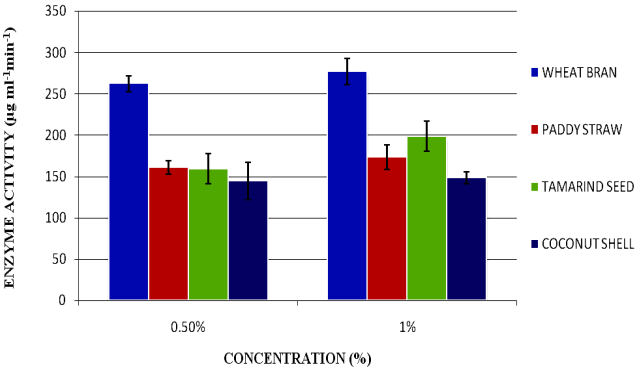

Figure V: Effect of ammonium sulfate concentration on cellulase production

3.6 Gel diffusion assay for detection of cellulase activity of precipitated enzyme

The largest zone of cellulose hydrolysis was observed with the enzyme precipitated with 40% ammonium sulfate, measuring 1.60cm which is comparable with the diameter of the cellulase standard which measured 1.64cm.

4. Discussion

Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes. Habitats that contain these substrates are the best sources in which to find these microorganisms[25]. Cow dung was selected as a source of diverse group of cellulolytic microorganisms owing to diet of the ruminants which consists of high amounts of cellulosic matter[26]. Further, its wide availability, ease of processing and cost effectiveness also plays important role for its selection[25]. The bacteria isolated from cow dung were examined for their morphological and biochemical characteristics and were further screened for the ability to produce cellulase. Growth characterization is essential as it influences the growth rate and hence enzyme production. Optimum pH and temperature conditions for the growth of the isolated *Bacillus sp.* were determined by turbidometry. The isolate was found to grow well at a pH of 8.0 showing that the bacteria are capable of growing at alkaline pH. The highest growth of the bacteria was at 40°C.

Cellulase was produced using four different agro- based wastes- wheat bran, paddy straw, tamarind seed powder and coconut shell powder. cellulase enzyme production using wheat bran and paddy straw is well documented and therefore can be used to compare with other novel substrates. The optimization of the cellulase production was carried out with all four substrates. A comparison of the enzyme yields with different substrates was made. Wheat bran was found to be the best substrate for

cellulase production followed by paddy straw. The novel substrates- tamarind seed powder and coconut shell powder were found to be very good competitors to the conventional substrate- paddy straw. Cellulase production using pretreated and untreated coconut shell powder was compared in order to determine the effect of pretreatment on cellulase production. Untreated coconut shell was found to better support the growth of the bacteria and thereby better production of the enzyme. Production of the enzyme varies with different substrates at different pH and temperature. Both inorganic and organic source of nitrogen were found to be suitable for optimization. The enzyme purified using 40% ammonium sulfate showed highest cellulase activity.

Conclusion

In the present study, potential cellulase producing bacteria were isolated from cow dung and the best producer of cellulase was identified by Congo red test. It is well characterized from earlier work that Bacillus sp. are good producers of the enzyme cellulase. The enzyme was partially purified and the activity of the purified enzyme was determined by gel diffusion assay. The study showed that the novel substrates- tamarind seed powder and coconut shell powder, under optimized conditions can be used as promising substrates for the large scale production of cellulase.

Conflict of interest

The author's declares none.

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