

Original Research Article

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Production of Cellulase from *Bacillus subtilis* under Solid- State Fermentation Using Fiber Wastes of Palmyra Palm

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ABSTRACT

In the present study, the production of cellulase has been carried out using novel natural waste as substrates by *Bacillus subtilis* isolated from slaughter house waste rumen fluid. *Bacillus subtilis* was screened for cellulase production by Congo red assay. pH and temperature were optimized. Agricultural wastes such as Hay, fiber waste of palmyra palm and banana bracts were used as a substrate for cellulase production. Substrates with or without pretreatment using NaOH solution were used for solid state fermentation. After 48hrs of fermentation, extracts were drawn and tested for enzyme activity by DNSA method and total protein content by Lowry's method. Cellulase activity was found to be 0.1519, 0.0759 and 0.05557 $\mu\text{mol ml}^{-1}\text{min}^{-1}$ when fibers of palmyra palm, hay and banana bracts were used as substrates. Raw cellulose content was found to be 37%, 32% and 23.2% in fibers of palmyra palm, hay and banana bracts respectively. Fibers of palmyra palm were found to be having maximum enzyme activity of 0.1519 $\mu\text{mol ml}^{-1}\text{min}^{-1}$ at pH 7, at 48hrs of incubation.

Keywords

Cellulase, *Bacillus subtilis*, Palmyra palm, Solid state fermentation, Agricultural waste.

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Introduction

Cellulases are crucial enzyme synthesized by a large variety of microorganisms including bacteria, fungi and some protozoans during their growth on cellulosic biomass. The cellulase is a family of at least 3 groups of enzymes: Endo-glucanase (EC 3.2.1.4), Exo-glucanase (EC 3.2.1.91) and β -glycosidases (EC 3.2.1.21). Cellulases hydrolyse the cellulose and convert it into monosaccharides (glucose, maltose). This process of breakdown is called Cellulolysis (Haigler *et al.*, 1985). Mechanistically, endoglucanase acts on the internal O-glycosidic bonds,

whereas exoglucanase acts on the ends of the cellulose chain thereby releasing β -cellobiose as an end product; and the β -cellobiose acts on the disaccharides and produce glucose. The mechanism of cellulose degradation by anaerobic bacteria is different from aerobic cellulolytic bacteria.

Cellulases have been used for both academic and industrial purpose (Ramesh chander kumahad *et al.*, 2011). The low production cost and good enzymatic activity increase the value of cellulase for commercial use. The

cellulase enzyme finds widespread applications in various industries which include textile, paper and pulp, food and agriculture. Cellulases are also helpful in controlling plant diseases.

Cellulases play an essential role in the Animal feed industry, the nutritional value of an animal feed can be increased by the pretreatment with cellulases. Apart from the major applications, cellulases are used for olive oil extraction, manufacture of cellulase based detergents and in bioconversion of ethanol and other organic solvents.

Increased uses of cellulases are the reason for high cost and greater demand. The high costs are due to the cost of the substrate. The utilization of cheaper substrates reduces both substrate cost and usage of costly chemicals for the fermentation procedure (Zhang *et al.*, 2006).

Many of the agricultural wastes are the cellulosic waste so they can be used as substrates for the production of highly demand cellulase. Most of the agricultural wastes are the renewable source of substrates and inexpensive.

The lignocellulosic resources are saw dust, sugarcane baggase, coir pith, rice hulls, husks, vegetable and fruit peels, corn cobs, woody crops, forest and agro-residues (Ogbuagu *et al.*, 2013). The cell wall of forages contains 24 to 36% cellulose and 4.3 to 8% lignin, the presence of these components slower the degradation rates. Moreover, cellulases contribute 8% of demand in the global enzyme. The cost of cellulase production is mainly because of the substrates used, hence the present study has been undertaken to produce cellulase by solid state fermentation using cheaper substrate fiber waste of palmyra palm (Sangrila Sadhu and Tushar Kanti Maiti, 2013).

Materials and Methods

Isolation and screening of cellulase producers by congo red assay method

A cellulase producing *Bacillus subtilis* was isolated from Rumen fluid collected from slaughter house by using streak plate method. An efficient cellulase producer is determined by Congo red assay (Gohel *et al.*, 2014). The isolated colony of *B. subtilis* was screened for Cellulase production. The screening was done by well diffusion method on Carboxymethyl cellulose (CMC) agar plates. Bacterial cultures with CFU 22×10^{-4} per ml were inoculated on wells of each plate. The plates were incubated at 37°C for 48hours in an incubator. After the incubation period, the plates were flooded with 0.1% Congo red and then it was left undisturbed for 20 minutes. The formation of clear zone was visualized by destaining the plates with 1M NaCl solution and then it was left undisturbed for another 15 minutes. The clear halo zones were observed around the colony.

Optimization of cellulase production with different substrates

Three lignocellulosic substrates were taken for the cellulase production by solid state fermentation. The substrates chosen were Hay, fiber waste of Palmyra palm and Banana bracts. These substrates were collected from a local market in Chennai. Each of the substrates were cut into small pieces and dried in a hot-air oven to remove the moisture content in the substrates and stored in an air tight cover.

Determination of raw cellulose content in substrates

Raw cellulose contents in the taken substrates were determined by using Weendize method (Henneberg, 1975). 1gm of each substrate was taken separately in a 200ml beaker.

1.25% of 200ml Sulphuric acid was added and boiled for 30 minutes. After 30 minutes, each sample was filtered using a muslin cloth and washed with distilled water to neutralize the pH. 1.25% of Sodium Hydroxide solution added to each sample and was boiled for 30 minutes. After boiling, each sample was filtered and washed with hot distilled water. The solid residue was washed with ethyl alcohol. The residue was dried at 105°C. Then it was cooled and weighed. The addition of Sulphuric acid removes all monosaccharides, and Sodium hydroxide removes proteins by hydrolysis and fats by saponification. Ethyl alcohol removes dyes, tannins, fat marks and raw ash complex. The percentage content of cellulose in each substrate was calculated by the following formula:

$$\% \text{ of cellulose content} = \frac{\text{Final volume}}{\text{Initial volume}} \times 100$$

Optimization of pH for cellulase production

Optimization was carried out in a basal salt medium containing carboxymethyl cellulose. The usual temperature of 37°C and 72 hours of incubation period was used. Various pHs taken for the study were pH 4, 5, 6, 7 and 8.

Optimization of incubation time for cellulase production

In view of the fact that cellulase is a primary metabolite; yield will be maximum within 72 hours. To study about the optimum incubation time, different incubation times 24, 48 72 hours were employed. The flasks were kept in an incubator shaker at 120rpm at 37°C.

Assay of cellulase

After the incubation period of every optimization, the culture flasks were centrifuged at 5000rpm for 15 minutes. The

pellet was discarded and the cell-free supernatant was taken as crude enzyme and it was subjected to enzyme assay. The activity of cellulase was assayed using DNSA method (Jahir Alam Khan and Sumit Kumar Singh, 2011) and total protein content by Lowry's method (Lowry, 1951). The equation used for enzyme activity determination is:

$$\text{Enzyme activity} = \frac{\text{Amount of product formed}}{\text{molecular weight of CMC} \times \text{Vol.of enzyme (ml)} \times \text{Incubation time (mins)}}$$

Based on maximum units of enzyme activity determined from DNSA method, suitable pH and incubation time was selected. These essential parameters were maintained in the solid state fermentation.

Solid state fermentation (SSF) without pretreatment

Air dried raw substrates were directly taken for the fermentation process without any pretreatment. The moisture content of the substrate was maintained at 1:10 ratio. 1gm of each substrate (Hay, fibers of Palmyra palm, Banana bracts) was weighed and taken in 50ml Erlenmeyer's flask. 10ml of autoclaved distilled water was added to all the three flasks and the pH of each flask was adjusted to 6. The contents were autoclaved and bacterial culture with CFU 22×10^4 per ml was inoculated in each. Inoculated flasks were maintained at 37°C in an incubator shaker at 120rpm for 2 days (72 hours). After 72 hours of incubation, produced enzyme was extracted from the culture flasks.

Solid state fermentation (ssf) with pretreatment

Alkali pretreatment was done to remove the lignin barrier present in the substrates. Alkali pretreated substrates were dried and used for fermentation process (Moses *et al.*, 2012).

2gms of each substrate was measured and taken in a 250ml beaker. 50ml of 1.25% Sodium hydroxide solution was added to each and boiled at 80°C for 20 minutes. The contents were filtered and washed with distilled water for several times to neutralize the pH of the substrate. The residue was dried in hot air oven at 80°C for 2 hours.

1gm of oven dried substrates was weighed and taken in an Erlenmeyer's flask each. 10ml of autoclaved distilled water was added to each flask and pH was adjusted to 6. Flasks with the substrates were autoclaved and bacterial culture with CFU 22×10^4 per ml was inoculated to each flask. Then the flasks were maintained at 37°C in an incubator shaker at 120rpm for 2 days (72 hours). After an incubation period of 72hrs, extraction of cellulase was carried out.

Extraction of cellulase

An ideal solvent found for this extraction is 0.2M Acetate buffer at pH 4.8 and 10 minutes of an extraction time was found to be best suited. 30ml of 0.2M acetate buffer was added to each flask and kept in a shaker for 10 minutes at 120rpm.

The mixture was filtered using a sterile muslin cloth and the filtrate collected was centrifuged for 15 minutes at 5000rpm to obtain a cell free supernatant. Pellet was discarded and the supernatant was stored which was taken as a crude enzyme. A cell free culture supernatant was a crude enzyme stored at 4°C and it was taken for cellulase assay tests (Pirota *et al.*, 2013).

Assay of cellulase from substrates

3, 5 Dinitrosalicylic acid reagent method was used to determine the cellulase activity, based on activity unit's best substrate was found. Lowry's method was performed to determine the total protein content present in the crude

enzyme.

Results and Discussion

Congo red test

Zone of hydrolysis produced by *B.subtilis* was 2.5 cm and is shown in figure 1.

Raw cellulose content determination

Raw cellulose content of hay, palmyra palm and banana bracts was found to be 32%, 37% and 23.2% respectively is shown in figure 2. Maximum cellulose content was found to be present in fibre waste of palmyra palm when compared to hay and banana bracts.

Production of cellulase at different pH is given in table 1. Maximum production of cellulase was obtained at pH 7 and 8; $0.0177 (\mu\text{mol ml}^{-1}\text{min}^{-1})$.

Cellulase production at different incubation time is given in table 2. Maximum production of cellulase was obtained at 48hrs of incubation; $0.1494 (\mu\text{mol ml}^{-1}\text{min}^{-1})$.

Solid state fermentation with and without pretreatment

Enzyme activity obtained after solid state fermentation when hay, fibre waste of palmyra palm and banana bracts were used as substrates with and without pretreatment is given in table 3. Enzyme activity was found to be maximum, when fibers of palmyra palm were used as a substrate in both with and without pretreatment. When banana bracts were used as substrate with pretreatment the enzyme activity was found to be higher when compared to without pretreatment.

When hay was pretreated the enzyme activity was found to be reduced. The optimum pH for *B.subtilis* was found to be 6.

Table.1 pH optimization – DNSA method and Lowry’s method

pH of media	Reducing sugar concentration ($\mu\text{g ml}^{-1}$)	Enzyme activity units ($\mu\text{mol ml}^{-1}\text{min}^{-1}$)	Total protein content ($\mu\text{g ml}^{-1}$)
4	90	0.0037	450
5	280	0.0118	320
6	560	0.0236	410
7	420	0.0177	320
8	420	0.0177	325

Table.2 Incubation time -DNSA method and Lowry’s method

Incubation time in hours	Reducing sugar concentration ($\mu\text{g ml}^{-1}$)	Enzyme activity units ($\mu\text{mol ml}^{-1}\text{min}^{-1}$)	Total protein content ($\mu\text{g ml}^{-1}$)
24	570	0.1443	425
48	590	0.1494	540
72	530	0.1342	340

Table.3 Raw and pretreated substrate – DNSA method and Lowry’s method

S.no	Name of the substrate	Reducing sugar concentration ($\mu\text{g ml}^{-1}$)		Enzyme activity units ($\mu\text{mol ml}^{-1}\text{min}^{-1}$)		Total protein content ($\mu\text{g ml}^{-1}$)	
		Without pretreatment	With pretreatment	Without pretreatment	With pretreatment	Without pretreatment	With pretreatment
1.	Hay	300	260	0.0759	0.0658	330	355
2.	Fibers of Palmyra palm	600	600	0.1519	0.1519	355	600
3.	Banana bracts	220	420	0.0557	0.1063	260	1385

Fig.1 *B. subtilis* showing maximum zone of hydrolysis

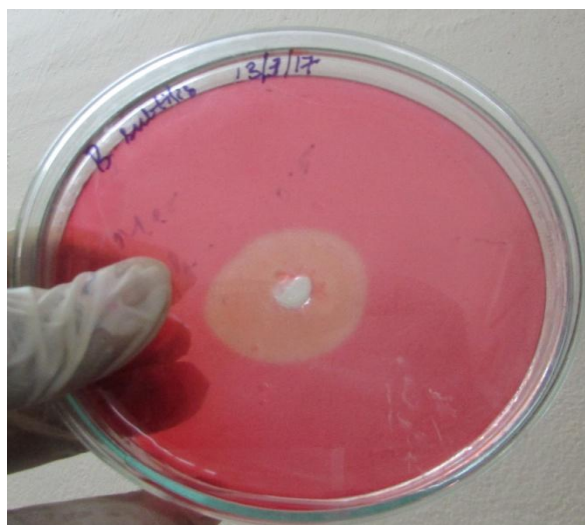
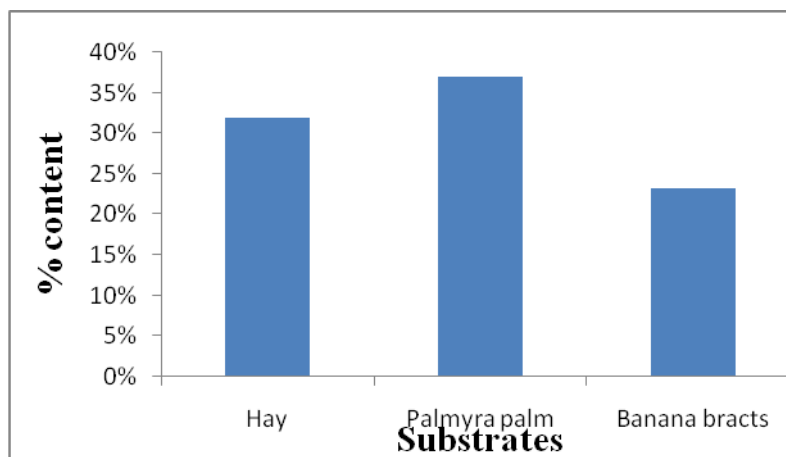


Fig.2 Raw cellulose content of substrates



It has the reducing sugar concentration of 560 μ g per ml and it showed the maximum enzyme activity units of 0.0236 μ mol ml⁻¹min⁻¹. The pH range 6.5 to 7.5 was obtained as an optimum pH and enzyme activity unit was 12 IU per ml for *B. subtilis* isolated from agricultural fields (Vipul Verma *et al.*, 2012). *B. subtilis* isolated from cow dung showed an optimum pH at 7 and the enzyme activity was 31.87 U per ml (Saraswathi Bhai *et al.*, 2012). This shows that the maximum production of cellulase is at the pH 6. In the present study, the optimum pH was found to be 7 and 8.

The optimum incubation time required for producing cellulase was 48 hours. It has the maximum enzyme activity unit 0.1419 μ mol ml⁻¹min⁻¹. This shows that 48 hours of incubation time is suitable for the cellulase production. Most of the *Bacillus sp.* took 48 hours for the maximum production of cellulase with enzyme activity of 15 IU per ml (Vipul verma *et al.*, 2012). *B.licheniformis* also possesses optimal incubation time as 48 hours (Bala Kumaran *et al.*, 2015).

Among the three substrates (hay, palmyra palm, banana bracts), the fibers of palmyra palm showed the maximum activity unit 0.1519 μ mol ml⁻¹min⁻¹ with reducing sugar concentration of 600 μ g per ml. Pretreated hay

showed less enzyme activity units when compared with not pretreated substrates and fibers of palmyra palm exhibited same enzyme activity unit even after the pretreatment. But banana bracts showed an increase in its enzyme activity when pretreated substrates were used. In overall comparison, fibers of palmyra palm showed the maximum enzyme activity in both fermentation process Based on the enzyme kinetics, fibers of Palmyra palm were selected as a suitable substrate for the production of cellulase by *B. subtilis*. Other substrates like wheat bran showed the highest yield of 20.96 U per ml (Saraswathi Bhai *et al.*, 2012). When *Jatropha* deoiled seed cake was used to produce endoglucanase from *Thermoascus* showed an enzyme activity of 45U mg⁻¹ (Bhaumik *et al.*, 2015).

The present study revealed that palmyra palm waste can be used as substrate with pretreatment using NaOH for maximum cellulase activity. Banana bracts can also be used as substrate without pretreatment.

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