ISOLATION AND SCREENING OF THERMOPHILIC CELLULOLYTIC BACTERIA FROM COMPOST PILES

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Abstract: Cellulose, a major polysaccharide found in agricultural residues and industrial and municipal wastes. In the present study thermophilic cellulolytic microorganisms were isolated. The isolates were tested for their cellulytic activity. The enzyme production from potent isolates was optimised using cellulose basal broth medium. Activity of partially purified enzyme was determined. The most potent thermophilic cellulolytic isolates were identified as *Bacillus subtilis*. The crude cellulase enzyme concentrated at 80-85% ammonium sulfate produced highest zone of hydrolysis. The enzymatic degradation of cellulose waste has been suggested as a feasible alternative for the conversion of lignocelluloses substrate into fermentable sugars and application for biofuel production.

Keywords: Thermophilic cellulolytic bacteria; Cellulase enzyme; Compost piles.

INTRODUCTION

Cellulose is biologically renewable resource abundantly found in agriculture waste. The cellulosic waste materialcan be hydrolysed to glucose and other soluble sugars by using cellulase enzymes of bacteria and fungi. The reducing sugars obtained can be further used for the production of ethanol as biofuel (Eriksson *et al*., 2002). The potential cellulose producing bacteria are Cellulomonas, Pseudomonas, Thermoactinomycetes and *Bacillus* spp.(Godana, 2007).

It has been estimated that 7.2×10^{11} tonnes of cellulose is reserved in plants and that the yearly production of cellulose is 4×10^{10} tonnes (Coughlan, 1985). Cellulases include three main types of enzymes, endoglucanases, cellobiohydrolases or exoglucanases and â-glucosidases. These enzymes can either be free or grouped in a multicomponent enzyme complex (cellulosome) found in anaerobic cellulolytic bacteria (Mosier *et al*., 1999).

Enzymatic hydrolysis is an economic process in the conversion of cellulose to easily fermentable low cost sugars (Muthuvelayudham and Viruthagiri, 2006). Thermo philic filamentus fungi, and actinomycetes are widely used for industrial production of specific, stable and active enzymes (Mach and Zeilinger, 2003; Haki and Rakshit, 2003).

Present study aimed to isolate and screen potential thermophilic cellulolytic microorganisms from compost piles.

METHODOLOGY

The present study was conducted at the laboratory of Nepal Academy of Science and Technology (NAST) from January 2009 to October 2010.

Enrichment, Isolation and Screening of Thermophilic Cellulytic Bacteria

A total of 13 different compost samples (temperature ≥ 50 °C) were collected in sterile containers. The compost piles were air dry and heated at 55^0C for 1 week and air drying to reduce the mesophiles and anaerobic isolates. Two protocols were used for isolation of thermophilic cellulase producer's bacteria. One gm of compost piles was serially diluted in order to reduce the initial number of microorganisms. Appropriate dilution was then both direct spread plated on CMC agar medium or enriched Cellulose broth and plating (spread) on (CMC) Agar. All incubations were done at 55°C for 2-4 weeks with shaking at 120 rpm in a controlled environment shaker (Ibrahim and El-diwany, 2007).

Screening of Potent Cellulytic Thermophilic Isolates

1% congo red indicator (El-Sersy et al., 2010) was applied for screening of cellulolytic bacterial isolates. The plates were flooded with 1% congo red indicator and left for 15mins followed by adding 1M NaCl solution and again left for another 15 min. then the development of halo zone around the colony indicated the cellulose hydrolysis. If not clear, 0.1N HCL was added to make further clearer zone (Wood and Bhat, 1988 and El-Sersy *et al*., 2010).

Identification of Thermotolerent and Thermophilic Cellulytic Microorganism

Representative bacterial isolates exhibiting high cellulytic activity were selected for identification according to method described by Bergey's Manuals of Systematic

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Bacteriology and Determinative Bacteriology (Ghazifard *et al*., 2001; Altai *et al*., 1989).

Enzyme Production and activity determination

Batch fermentation was adopted for enzyme production was done by inoculating 5 ml of each pre-fermenter inoculum into 500 ml-Erlenmeyer flasks containing the same 100 ml cellulose basal broth medium and maintained the final inoculums size 5%. The flasks were shaken at 150 rpm and incubated at 50^0C for 5- 7 days (Omojasola *et al.*, 2008). The submerged cultures were carried out in duplicate. The previously studied different Basal mediums were tested for maximum production of cellulase enzyme. The fermentation was monitored for cellulase activity, reducing sugar and protein content**.** Activities of cellulase enzyme system secreted into the culture medium of potent isolates were estimated in accordance with following methods listed by Wood and Bhat (1988). The evaluation of crude enzyme Cellulolytic activity was carried out for measuring zone of hydrolysis on 1% CMC agar medium by Plate diffusion assay method (Oyekola, 2003). Fermentation was monitored day-wise by estimating reducing sugars by DNS method (Miller, 1959).

Enzyme activity was determined spectrophotometrically as adapted from the recommendations of the Commission of Biotechnology (Wood and Bhat, 1988).

Protein estimation

The protein content of the culture supernatant was determined by Bradford *et al.*, (1976).

Partial Purification and Evaluation of Cellulase Enzyme

Partial Purification of the enzyme from culture supernatant was done by Ammonium sulfate concentration method (Aboul-Enein *et al*., 2010; Wood and Bhat (1988).

Characterization of Enzyme

Thermal Stability**:**

To determine the thermal stability of the enzyme, the respective isolates of enzyme was incubated in 0.05M phosphate buffer of pH 7.0 and 0.05 M sodium citrate pH 4.8 for 1 hour at temperature ranging from 30-80ºC (Oyekola, 2003; Ariffin *et al*., 2006).

Table 1: Colony morphology and Microscopic characteristics of cellulytic Bacterial isolates.

Presumptive genera	Colonies morphology	Microscopic characteristics
Amphibacillus spp.	circular, convex and white, 1-3mm	A facultatively anaerobic, alkaliphilic, spore-forming, Gram-positive-staining rod, endospore are oval and central, somewhat swelling cell, motile by peritrichous flagella.
Bacillus licheniformis	Rhizoid convex 0.5 cm rough dry opaque, yellow	Rough and Smooth forms, mucoid, short baciili and also filamentous type , flagellate ,non sporing
Bacillus spp.	Round translucent colony with smooth edges. Gram-positive rods with endospores. Motile.	Rod shaped and straight, 0.5-2.5*1.2-10um in size are often arrane in pairs or chain, with rounded or square end. Gram positive, endospore are oval or sometime round or cylindrical, motile by peritrichous flagella.
Bacillus subtilis	Irregular raised 1.0cm rough / dry opaque white	Rod-shaped Gram positive bacteria , motile by peritrichous flagella and aerobic rod-shaped, and has the ability to form a tough, endospore
Cellulomonas cellulans	Colonies are, circular, convex, white, and glistening	In young culture, slender, irregular rods, 0.5-0.6*2-5um in size are straight or slightly curve, some rods are in pairs at an angle to each other giving v formation, rods occasionally slow branching, but no mycelium is formed. In old culture- rods are usually short, afew cocci may occur , Gram positive easily decolorized, often motile by one or two flagella, non sporing , non acid fast.
Clostridium spp.	Circular raised 0.7cm, smooth glistering, mucoid, translucent cream	Circular raised colony with 0.7 cm diameter, non-motile, Gram-negative short rods.
Geobacillus spp.	Colonies are round, convex, mucoid and colorless.	Aerobic or facultatively anaerobic, rod-shaped, either occurring short chains and motile by means of peritrichous flagella, Gram positive bacterium, sporulating rods with ellipsoidal endospores.
Penibacillus spp.	Colonies on are circular, flat, white/cream, opaque and usually 1–3 mm in diameter	Aerobic or facultatively anaerobic, Spore-forming rods, 0.8–0.9 µm wide and 4.0–4.2 µm long. Gram-variable., rods .Motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia and occupy a subterminal position in the cell.

Table 2: Cellulytic activity of bacterial isolates

S.N.	Isolates	Cellulytic zone of hydrolysis (well size 6mm)
1	*Bacillus subtilis**	26
2	*Cellulomonas cellulans***	22
3	*Geobacillus* spp.**	24
4	*Penibacillus* spp.*	23
5	*Bacillus* spp.**	25
6	*Cellulomonas cellulans**	25
7	*Bacillus licheniformis***	25
8	*Bacillus* spp*	24
9	*Bacillus* spp*	24
10	*Penibacillus* spp.*	21
11	? *Clostridium* spp.*	22
12	*Bacillus* spp.**	23
13	? *Clostridium* spp.**	21
14	*Bacillus subtilis***	25
15	*Bacillus* spp.*	22
16	*Bacillus subtilis***	25
17	*Amphibacillus* spp. **	20
18	*Cellulomonas cellulans***	25
19	*Bacillus* spp. **	23
20	*Amphibacillus* spp. **	21
21	*Bacillus subtilis**	26
22	*Geobacillus* spp.*	22
23	*Bacillus* spp. **	24
24	*Bacillus* spp. **	23
25	*Penibacillus* spp. **	22

**= Thermophilic and **= Thermotolerant ?= suspected*

Effect of Temperature:

The temperature profile between 30^{0}C and 80°C, for Cellulase activity, at optimum pH was determined. The soluble enzyme extract was incubated with the substrate (CMC) at various temperatures (30-80°C) (Oyekola, 2003 and Ray *et al.*, 2007; Ariffin *et al*., 2006).

Effect of pH:

In order to determine the optimum assay pH, for cellulase activity, the assay was carried out at different buffers used at various pH were: 0.05 M sodium acetate (pH 3-4.5), 0.05 M sodium citrate (pH 5-5.5) and 0.05 M sodium phosphate buffer (pH 6-8) (Oyekola, 2003; Ray *et al.*, 2007; Ariffin *et al*., 2006).

RESULT

In the present study 10 thermophilic and 15 thermotolerant bacteria were isolated from compost piles. They were identified as *Bacillus subtilis, Bacillus licheniformis, Bacillus* spp., *Cellulomonas cellulans, Geobacillus* spp. and *Penibacillus* spp. were common bacteria. The most potent thermophilic cellulolytic isolates was *Bacillus subtilis* (Table 1) McCaig *et al*., (2001); Song *et al*., (2001).

Production Optimization of Cellulase Enzyme

The *Bacillus subtilis* produced maximum reducing sugar and also showed highest zone of hydrolysis as 21mm in 8th day at optimal cellulose basal medium.

In all four different cellulose basal media used for enzyme production *Bacillus subtilis*, produced highest enzyme activity in the medium as described by Ray *et al*., (2007). The zone of hydrolysis was 30 mm.

Optimum pH for maximum activity of Partial Purification of Enzyme

The highest cellulolytic activity of crude enzyme (1:10 diluted) was given by the residue obtained by at 80 %ammonium sulfate precipitation at 4^{0}C. protein concentration of the residue was 0.865 mg/ml (Aboul-Enein et al., 2010)

The enzyme activity of *Bacillus subtilis*, was maximum at pH 7.2 at 50^{0}C. However, *Bacillus subtilis*, retained its activity from 6.6 upto 9.0 pH.

Thermostability of enzyme

Cellulase enzyme activity of *Bacillus subtilis* was optimum at 50^{0}C. However, the result was similar as reported previously (Marques *et al*., 2003 and Murashima *et al*., 2002).

DISCUSSION

Bacillus subtilis, was potent thermophilic cellulytic bacteria. Cellulase enzyme activity of *B. subtilis* was optimum at pH 7.2 at 50^{0}C at 1% CMC substrate concentration. The potential enzymatic degradation of cellulosic wastes by the thermotolent cellulase enzymes has been suggested as a feasible alternative for the conversion of lignocellulosics into fermentable sugars and fuel ethanol.

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