Production and partial purification of cellulose by *Basillus subtilis* Fumigatus fermented in coir waste and sawdust

S. ARUNKUMAR, V. SIVAKUMARI, M. MUTHUSELVAM AND N.V. BASKAR

Asian Journal of Environmental Science, Vol. 3 No. 2 : 123-127 (Dec., 2008 to May, 2009)

See end of the article for authors' affiliations

Correspondence to :
V. SHIVAKUMARI
Department of Environmental and Herbal Sciences, Tamil University, THANJAVUR (T.N.) INDIA

SUMMARY

Basillus subtilis* was used as fermentative organism for the production of cellulose enzyme using cheap substrate such as coir waste and sawdust. Maximum cellulose enzyme productivity was noted on coir waste compared with saw waste. Cellulose productivity was optimized in various physico-chemical parameters, such as pH, temperature, carbon and nitrogen sources. Cellulose enzyme protein fraction was analyzed by SDS-PAGE method. Coir waste can be used as the substrate for the large scale production of cellulose enzyme using *Basillus subtilis.

Key words : *Basillus subtilis*, Cellulose enzyme, SDS-PAGE, Coir waste and sawdust.

Accepted : September, 2008

Cellulose is commonly degraded by an enzyme called cellulose. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Bahkali, 1996; Magnelli and Forchiassin, 1999; Immanuel *et al*., 2006). Although a large number of microorganisms are capable of degrading cellulose, only a few of these produce significant quantities of cell free enzymes capable of completely hydrolyzing crystalline cellulose *in vitro*. Fungi are the main cellulose producing microorganisms, though a few bacteria and actinimycetes have also been reported to yield cellulose activity.

The specific cellulolytic activity shown by the bacterial species is found to be depending on the source of occurrence. Some features of cellulosic materials are known to inhabit their degradation / bioconversion. There are degree of crystalinity, lingnification and the capillary structure of cellulose to cellulolytic enzymes and other hydrolytic agents (Fan *et al.*, 1987). However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported (Kumakura, 1997; Kanosh *et al.*, 1999).

The bioconvertion of various complex cellulosic waste materials such as baggase (Kanosh *et al.*, 1999) have been reported. *Basillus subtilis* has been used for a bioware stimulant during project SHAD. Hence, the present study was carried out to determine the cellulolyticenzyme activity of bacterium, *Basillus subtilis* against coir waste and saw dust as carbohydrate source.

MATERIALS AND METHODS

The strain of *Bacillus subtilis* MTCC 1305 used in the present study was obtained from IMTECH at Chandigarh. The organism was cultured and maintained on Czapek medium for further study. The raw substrates were sun dried individually to reduce the moisture content to make them more susceptible for crushing. The crushed substrates were then sieved individually to get powder form. Then the substrates were soaked individually in 1% sodium hydroxide solution (NaOH) in the ratio 1: 10 (substrate: solution) for two hours at room temperature. After which, they were washed for free of chemicals and autoclaved at 121°C for one hour. The treated substrates were then filtered and washed with distilled water until the wash water became neutral (Gharpuray *et al.*, 1983).

The cultures of *Bacillus subtilis* were maintained as stock culture on Czapek- Dox agar slants. They were grown at 37°C for 24 hours and stored at 4°C for regular sub-culturing. 100 ml of inoculum was prepared for each culture using Czapek- Dox broth in 250 ml flasks. The inoculum was kept in shaker (200 rpm) at 37°C for 24 hrs before it was used for the fermentation process. To 100 ml of the optimized culture medium, 10ml of broth culture was inoculated under controlled conditions. Then it was kept in a shaker (200

rpm) at 37°C for a day. Simultaneously, separate media were prepared for coir as well as sawdust substrates.

The total amount of reducing sugars in 1ml supernatant was determined by modified Dinitro salicylic method (DNS) and also Cellulose activity was determined by filter paper activity (FPA) method. Filter paper activity is a combined assay for Endoglucanase (Cx) and Exoglucanase (Ci) cellulose.

It is an alternative to Nelson-Somogyi method. It is simple sensitive and adoptable during handling of a large number of samples at a time. The culture filtrate was collected from the fermentation media by centrifugation. 1 ml of culture filtrate was taken in a test tube and it was equalized with 2ml of distilled water. To the prepared culture filtrate, 3 ml of DNS reagent was added. The contents in the test tubes were heated in a boiling water bath for 5 minutes. After heating, the contents were allowed to cool at room temperature. At the time of cooling, 7 ml of freshly prepared 40% sodium potassium tartarate solution was added. After cooling, the samples were read at 510 nm in a U.V. spectrophotometer. The amount of reducing sugar was determined using a standard graph.

It is a combined assay for endo and exo α-1, 4 glucanase. The substrate used was Whatman No. 1 filter paper. 2 ml of crystalline cellulose solution was taken in a test tube [filter paper – (50 mg) and dissolved in 0.2 M sodium acetate buffer (pH 5.5)]. To this tube, 0.5 ml of the culture filtrate was added (enzyme solution). The mixture was incubated at 35°C for one hour and the reaction was terminated by adding 2 ml of DNS reagent. Then it was heated in a boiling water bath for 5 minutes and then 1 ml of potassium sodium tartarate (40%) was added to the warm tubes. The tubes were allowed to cool and the absorbance was read at 540 nm in a U.V. spectrophotometer. The enzyme production was expressed as the mg glucose released per minute per mg of protein.

The optimized media were prepared using the individual substrates and the pH was set at different levels such as 4, 5, 6, 7, 8 and 9by adding 1% NaOH and concentrated HCl. Then the media were autoclaved. Later, they were inoculated with broth culture and were placed in a shaker (150 rpm) at 37°C for 2 days. Simultaneously, for both the organisms and both the substrates, assay was carried out separately.

The optimized media were prepared individually by using the substrates and autoclaved. Later, it was inoculated with broth culture and was set at different temperatures 20, 30, 40 and 50°C, respectively. The effect of temperature on the production of cellulolytic enzyme was determined by growing the organisms at the above temperatures. Simultaneously, for the organisms and both the substrates, separate assay was carried out. The enzyme solution obtained from these two (pH and temperature) experiments was individually optimised based on Dinitro-salicyclic acid and filter paper activity methods as described earlier.

Different carbon sources (Carboxyl Methyl Cellulose (CMC), cotton, glycerol and filter paper) and nitrogen sources (Peptone, Tryptone, NH_4PO_4 and NH_4SO_4) weighing 750mg each, were added to 25 ml of the medium in separate 100 ml flasks and sterilized by autoclaving. Each flask incubated at 37°C in a rotary shaker at 250 rpm. Duplicate flasks were analysed at 24 hours intervals for 168 hours.

The dialysed enzyme samples from each source was analysed through PAGE using specific standard molecular marker. The gel obtained was photographed and scanned using a gel documentation system (SYNGENE, UK). Then the molecular weight of individual enzyme fractions was determined by referring the molecular weight of the marker. Separation of proteins was made by SDS-PAGE using a vertical slab gel electrophoresis apparatus. Each lane received 100µg of protein.

The slab gel unit was thoroughly cleared and dried. The gel plate was fixed with appropriate spacers on the gel. To avoid leakage, vacuum grease was applied on both sides of the spacer. The volume of the gel was measured with distilled water. The solution was poured into the plate up to the level such that 3cm gate was allowed for stacking gel. The air bubbles were removed and then added even layer of isobutanol on the top the separating solution, to get a flat surface on the top of the gel. Then allowed to polymerize for 30min. Then the isobutanol layer was removed and washed with distilled water.

The stacking gel solution was prepared. Then the comb was inserted in between the plates. The solution was poured carefully on the top of the separating gel and bulles were removed. After 30minuted, the bottom spacer was removed and the gel plate fixed into the electrophoresis apparatus.

The protein samples and standard protein markers were taken in eppendoff tube separately. Equal volume of sample buffer was added to the sample solution and boiled in a water bath at 100^0C for 3 minutes. The 100µl of above samples were loaded into each well and the equal amount of standard protein marker was loaded to any one of the well to compare the molecular weight of the sample proteins. Then electrophoresis buffer was added in anode and cathode chambers until the buffer touches the gel.

The power supply was connected and applied to 60V until the sample dye entered the separating gel and the increased the voltage at 100 till the sample band reached the end of separating gel. After reaching the sample band, the power supply was disconnected and the slab gel set up was removed. Then the glass plate was removed carefully.

The gel was placed in coomassie brilliant blue R-250 stains for 2 to 4 hours. The location of a protein in a gel was determined by coomassie brilliant blue staining. Then the gel was destained in destaining solution for 1-2 hrs until clear background was obtained. Then the gel was photographed immediately after the destaining. The protein bands were analysed by using a Gel Documentation System (CDS) linked to a Computerized Image Analyzer (Quality one Analyzing Software, BIORAD, USA). The gels were scanned in a scanning densitometer model GS300 (Hoefer Scientific Instruments, USA). The experiments were repeated 4 times and the results were reproducible.

The number and molecular masses of the comassie brilliant blue stained bands detected on the gel were determined with the Image Analyzer and the presence or absence of protein bands corresponding to polypeptides identified using SDS-PAGE was compiled into a data set. The results obtained in the present study were subjected to SD and One-Way ANOVA described.

RESULTS AND DISCUSSION

In the present study, *Bacillus subtilis* cellulose enzyme production was analyzed in cheap substrates like coir waste and sawdust. The cellulose enzyme production by *Bacillus subtilis* in varying pH using coir waste as substrate showed maximum at pH 6 (0.082 ± 0.002 IU ml^{-1}) and it was optimum, further when the pH level increased, the enzyme production was reduced, *i.e.* 0.072 ± 0.003 to 0.038 ± 0.004 IU ml^{-1} from pH 6–9, respectively (Table 1).

Table 1 : Cellulase enzyme activity (IU ml^{-1}) by *Bacillus subtilis* on coir waste and saw dust using DNS method

Sr. No.	pH Level	Enzyme activity (IU ml^{-1})	
		Coir waste	Sawdust
1.	4	0.032±0.003	0.021±0.001
2.	5	0.068±0.002	0.028±0.003
3.	6	0.082±0.002	0.032±0.002
4.	7	0.072±0.003	0.062±0.004
5.	8	0.062±0.002	0.058±0.002
6.	9	0.038±0.004	0.046±0.003

Bacillus subtilis grown in sawdust supplemented growth medium, it produced 0.032 ± 0.002 IU ml^{-1} cellulose enzyme at pH 6. But at pH 7, it produced 0.062 ± 0.004 IU ml^{-1} enzyme and it was the optimum, further when the pH level increased, the amount of enzyme production was significantly decreased (Table 2).

Table 2 : Cellulase enzyme activity (IU ml^{-1}) by *Bacillus subtilis* on coir waste and saw dust at different p^h using DNS method

Sr. No.	pH Level	Enzyme activity (IU ml^{-1})	Sawdust
		Coir waste	
1.	4	0.032±0.003	0.021±0.001
2.	5	0.068±0.002	0.028±0.003
3.	6	0.082±0.002	0.032±0.002
4.	7	0.072±0.003	0.062±0.004
5.	8	0.062±0.002	0.058±0.002
6.	9	0.038±0.004	0.046±0.003

The cellulose enzyme production by FPA method determined that *Bacillus subtilis* grown at coir waste as major carbon source in the growth medium showed an optimum production at pH 7 (0.288 ± 0.004 IU ml^{-1}), whereas at either sides, it was 0.120 ± 0.003, 0.177 ± 0.002, 0.254 ± 0.003 and 0.196 ± 0.003 at pH 5 to 6 and 8 to 9, respectively. *Bacillus subtilis* grown in saw dust supplemented medium showed the enzyme production at least level at pH 5 (0.080 ± 0.02 IU ml^{-1}), further it increased to an optimum at pH 7 (0.264 ± 0.002 IU ml^{-1}). Then, the enzyme production was gradually reduced to 0.240 ± 0.002 and 0.233 ± 0.003 IU ml^{-1} at pH 8 and 9, respectively (Table 3).

Table 3 : Cellulase enzyme activity (IU ml^{-1}) by *Bacillus subtilis* on coir waste and saw dust at different pH using FPA method

Sr. No.	pH Level	Enzyme activity (IU ml^{-1})	Sawdust
		Coir waste	
1.	4	0.092±0.003	0.190±0.002
2.	5	0.120±0.003	0.196±0.003
3.	6	0.177±0.002	0.257±0.002
4.	7	0.288±0.004	0.264±0.002
5.	8	0.254±0.003	0.240±0.004
6.	9	0.196±0.003	0.233±0.003

The results revealed that the enzyme production by *Bacillus subtilis* was less at 20°C (0.186 ± 0.004 IU ml^{-1}), it increased to 0.196 ± 0.003 IU ml^{-1} at 30°C and it reached a maximum of 0.258 ± 0.003 IU ml^{-1} at 40°C.

This is the optimum temperature and further the temperature increased, the enzyme production was significantly decreased (0.241 ± 0.002 IU ml^{-1}) at 50°C. The enzyme production by *Bacillus subtilis* was less at 20°C (0.162 ± 0.002 IU ml^{-1}), further it increased to 0.170 ± 0.005 and 0.204 ± 0.002 IU ml^{-1} at 30 and 40°C, respectively. Finally it reached to a maximum at 50°C (0.230 ± 0.003 IU ml^{-1}) (Table 4).

Table 4 : Cellulase enzyme activity (IU ml^{-1}) by *Bacillus subtilis* on coir waste and saw dust at different temperature (^{0}C) using DNS method

Sr. No.	Temperature (^{0}C)	Enzyme activity (IU ml^{-1})	
		Coir waste	Sawdust
1.	20	0.186±0.004	0.162±0.001
2.	30	0.196±0.003	0.170±0.005
3.	40	0.256±0.003	0.204±0.002
4.	50	0.241±0.002	0.230±0.003
5.	60	0.199±0.002	0.162±0.003

At 20°C, the enzyme production by *Bacillus subtilis* was 0.290 ± 0.005 IU ml^{-1} and it increased to 0.298 ± 0.005 IU ml^{-1} at 30°C. At 40°C, it reached its maximum of 0.338 ± 0.003 IU ml^{-1} and subsequently, the enzyme production was reduced to 0.278± 0.002 IUml^{-1} at 50°C. The *Bacillus subtilis* when grown in the growth medium supplemented with saw dust as substrate showed the maximum production of enzyme (0.340 ± 0.003 IU ml^{-1}) at 40°C, whereas at the temperature 20, 30 and 50°C, the enzyme production was at the rate of 0.271 ± 0.001, 0.318 ± 0.005 and 0.328 ± 0.002 IU ml^{-1}, respectively (Table 5).

Table 5 : Cellulase enzyme activity (IU ml^{-1}) by *Bacillus subtilis* on coir waste and saw dust at different temperature using FPA method

Sr. No.	Temperature (^{0}C)	Enzyme activity (IU ml^{-1})	
		Coir waste	Sawdust
1.	20	0.290±0.005	0.271±0.001
2.	30	0.298±0.005	0.318±0.005
3.	40	0.338±0.003	0.328±0.002
4.	50	0.278±0.002	0.340±0.003
5.	60	0.168±0.002	0.312±0.003

Enzyme induction was studied by using various defined substrates namely Carboxyl Methyl Cellulose (CMC), cotton, glycerol and filter paper. Among the four carbon source used cotton showed more per cent loss (up to 78%) followed by filter paper (71.98% than the CMC (48%) glycerol (32% to 58.81%). Maximum loss was observed with third day and fourth day followed by fifth day and sixth day (Table 6).

Table 6 : Cellulase enzyme activity (IU ml^{-1}) by *Bacillus subtilis* on coir waste and saw dust at different carbon sources using DNS method

Incubation days	Carbon substrates / Enzyme activity (UI ml^{-1})			
	Cotton	Filter paper	CMC	Glycerol
1	0.050	0.040	0.030	0.048
2.	0.054	0.052	0.032	0.051
3.	0.063	0.057	0.048	0.058
4.	0.063	0.071	0.045	0.057
5.	0.076	0.046	0.043	0.048
6.	0.078	0.046	0.043	0.032
7.	0.058	0.043	0.042	0.032

Table 3 shows the per cent loss of viscocity induced by the nitrogen sources such as NH_4SO_4, NH_4PO_4, peptone and tryptone by *B. subtilis*. CMCase was maximum induced by NH_4PO_4 from (32.21 to 66.82%) followed by NH_4SO_4 (up to 62.69%) but it induced the enzyme activity maximum at fifth day whereas tryptone at sixth day (up to 70.18%).

Peptone and tryptone induce maximum enzyme production from 72 hour to 144 hour (0.650 IUml^{-1}).Moderate induction observed with NH4PO4 whereas the NH_4SO_4 was maximum inducer from 24 hour to 48 hour intervals followed by a decline. Exoglucanase was highly induced by tryptone *i.e.* 1.33 IUml^{-1}on 120 hours whereas peptone, NH_4PO_4 and NH_4SO_4 were also a good inducer but it induces the enzyme activity at 144 hours (Table 7).

Table 7 : Cellulase Enzyme Activity (IU ml^{-1}) by *Bacillus subtilis* on coir waste and saw dust at different nitrogen sources using DNS method

Incubation Days	Per cent loss of viscosity at 120 minutes			
	NH_4PO_4	NH_4SO_4	Peptone	Tryptone
1.	0.036	0.046	0.036	0.040
2.	0.040	0.058	0.043	0.042
3.	0.062	0.060	0.042	0.046
4.	0.066	0.062	0.049	0.045
5.	0.056	0.055	0.061	0.048
6.	0.056	0.056	0.046	0.070
7.	0.052	0.052	0.046	0.048

The protein profile of cellulose produced by *Bacillus subtilis* using coir waste and sawdust as substrates. The protein fractions were determined by densitometric scanning. The relative mobility's of protein fractions in Rf value and corresponding molecular weight of each sample was determined. In the protein fractions of

Bacillus Cellulose two bands were obtain in the case of *Bacillus subtilis* grow on coir waste, the molecular weight of bands 39kDa and 26kDa, respectively. Similarly two bands also noted in the sawdust inoculated with *Bacillus subtilis* the molecular weight of 38kDa and 18kDa, respectively (Plate 1).

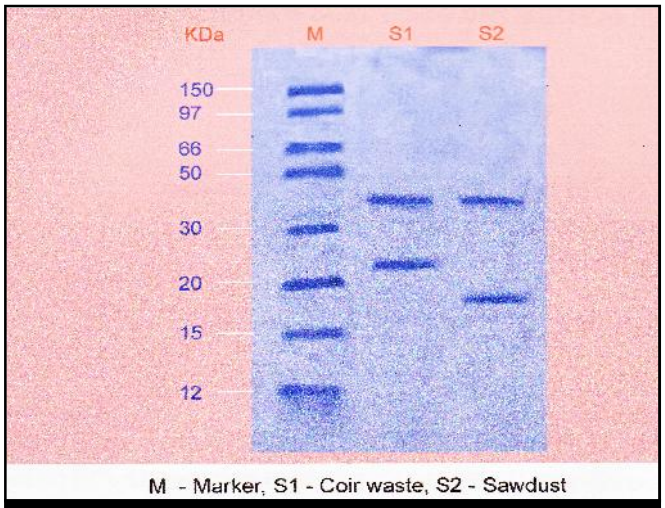

Plate 1 : Analysis of cellulose fraction of *Bacillus subtilis* on 10% SDS PAGE Electrophoresis

In the present study, the major cellulytic Bacteria strains for cellulose enzyme production. The natural sources of cellulose degradation are varied and these may be investigated by several investigators. For eg. termits are the best cellulose degrader in soil from the tropic to desert, they stir and mix with the aid of bacteria, protozoa and fungi, thereby they effectively recycle cellulose. The degradation activity of termites is by microbes present in their intestine by mushroom eg. *Lentinula edodes* etc.

Cellulose enzyme production accounts for 40% of cost in bioethanol synthesis. To reduce cost of production, the lignocellulosic substrates are used instead of synthetic cellulose due to their reasonable cost, high enzyme production capacity etc. The reduction in cost paves an economically easy way production of ethanol. It is an important issue to deal with the residue both the comprehensive utilization of lignocellulosic resources and for the prevention of environmental pollution.

Authors' affiliations

S. ARUNKUMAR, Department of Microbiology, Ponnaiyah Ramajayam College, THANJAVUR (T.N.) INDIA

M. MUTHUSELVAM AND N.V. BASKAR, Department of Microbiology, Marudhu Pandiyar College, THANJAVUR (T.N.) INDIA

REFERENCES

Bahkali, A.H. (1996). Influence of various carbohydrates on xylanase production by *V. tricorpus*. *Bioresource Technol.*, **33** (3) : 265-268.

Fan, L.T., Gharpuray, M.M., Lee, Y.H. (1987). *Cellulose hydrolysis*, Berlin, Germany, Springer-Verlag, **3** : 1 – 68.

Gharpuray, M.M., Lee, Y.H., Fan, L.T. (1983). Structural modification of lignocellulosic by treatment to enhance enzymatic hydrolysis. *Biotechnol.*, **25** : 157 – 170.

Immanuel, G., Dhanusa, R., Prema, P. and Palavesam, A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *Internat. J. Environ. Sci. Tech.*, **3** (1) : 25-34.

Kanosh, A.L., Essant, S.A. and Zeinat, A.M. (1999). Biodegradation and utilization of baggase with *Trichoderma ressei*. *Polym. Degrad. Stab.*, **62** : 273 – 276.

Kumakura, M. (1997). Preparation of immobilized cellulose bonds and their application to hydrolysis of cellulosic materials. *Proess. Biochem.*, **325** : 555-559.

Mangelli, P. and Forchiassin, F. (1999). Regulation of the cellulose complex production by *Saccobolus saccaboloides*, induction and repression by carbohydrates. *Mycologia.*, **91**(2): 359-364.

Miller, G.L., Blum, R., Glennon, W.E. and Burton, A.L. (1960). Measurement of carboxymethylcellulose activity. *Anal. Biochem.*, **2** : 127-132.

Ojumu, T., Solomon, V., Bamidele, O., Betiku, E., Layokun, S.K. and Amigun, B. (2003). Cellulose production by *Aspergillus flavus* Linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob. *African J. Biotechnol.*, **2** (6): 150-152.