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Optimization, Purification of Cellulase Produced From *Bacillus Subtilis Subsp. Inaquosorum* Under Solid State Fermentation And Its Potential Applications in Denim Industry

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Abstract: ***A bacterial isolate from soil samples contaminated with decaying lignocellulosic wastes and effluents of cotton industry was isolated for cellulase production and was identified as Bacillus subtilis subsp. inaquosorum after morphological, biochemical and phlogenetic analysis. Under optimized solid state fermentation conditions maximum cellulase production 71.15 IU/ml was obtained with wheat bran as carbon source in MA-1 mineral media and fructose and yeast extract as additives under 48 hr of production period at 37˚C, with 30% inoculum size. Moisture level of 1:2 (w/v) was found best for cellulase production. The crude cellulase so produced was further analysed for its potential applications in biostoning of denim. The purification of cellulase was carried out by ammonium sulfate precipitation followed by ion-exchange chromatography using DEAE-Sepharose and Sephadex G -150. The molecular weight of the purified carboxymethylcellulase (CMCase) was estimated to be about 32.5 kDa with the analysis of SDS-PAGE.***

Keywords: Bacillus subtilis, biostoning, cellulase, lignocellulosic waste, chromatography

1. Introduction

Cellulases have wide range of industrial applications in textile, laundry, pulp and paper, fruit juice extraction, and animal feed industries.[1] They also cause saccharification of lignocellulosic biomass to fermentable sugars, which, in turn can be used for production of bioethanol, lactic acid, single-cell protein, and other industrially important chemicals.[2–5] Cellulases are inducible enzymes synthesized by microorganisms during their growth on cellulosic material. These enzymes can either be free, particularly in aerobic microorganisms, or grouped in a multicomponent enzyme complex called ‘‘cellulosomes’’ in anaerobic cellulolytic bacteria.[6] Bacteria are now being widely explored for cellulase production because of their rapid growth, expression of multienzyme complexes, stability at extremes of temperature and pH, lesser feedback inhibition, capacity to colonize a wide variety of environmental niches, and ability to withstand varieties of environmental stress. They produce cellulases that are stable under extreme conditions, during bioconversion processes, which accelerate enzymatic hydrolysis, fermentation, and product recovery. These attributes induced a search for fast growing bacteria able to synthesize active cellulases under harsh conditions.[7,8] There are several reports on production of cellulases from bacteria such as Bacillus,[9] Clostridium,[10] Cellulomonas,[11] Ruminococcus,[10] and Streptomyces spp.[12] Bacillus spp. are most sought after as they produce diverse range of cellulases that are stable under extreme conditions.[13–17] Among Bacillus spp. B. subtilis continues to be a dominant workhorse due to its capacity to secrete large quantities of extracellular cellulolytic enzymes.[9,18,19,20] However, very few Bacillus spp. are reported to be able to produce lichenan and b-glucan hydrolyzing capacity along with CMCase activity at highly alkaline pH.[20,21] In our earlier study, we reported enhanced cellulase production by Bacillus subtilis by optimization under submerged fermentation . In the present report, we describe the purification and potential applications of cellulase from Bacillus subtilis subsp. inaquosorum in denim industry.

2. Material and Method

2.1. Chemicals and Reagents

Chemicals were obtained from Sigma Chemicals Co. All the chemicals and reagents used for the study were of analytical /microbiological grade and were obtained from commercial vendor.

2.2. Microorganism

Cellulase producing Bacillus subtilis subsp. inaquosorum was previously isolated using soil samples contaminated with decaying lignocellulosic wastes and effluents of cotton industry by enrichment method.(26)

2.3.Production of cellulase under solid state fermentation

For production of cellulase from *Bacillus subtilis* add 10 g of wheat bran in a 250-ml Erlenmeyer flask 20 ml mineral salt solution (g/l: MgSO4Æ7H2O, 0.2; K2HPO4, 0.4; pH 7.0) and sterilized by autoclaved. The flasks were brought to room temperature and inoculated with 10% (v/w) inoculum of an overnight culture and incubated at 37˚C for 72 h. The flasks were tapped at regular intervals in order to mix the contents.

2.4.Enzyme extraction

The enzyme from each flask was extracted twice with 0.1M glycine-NaOH buffer pH 8.0 (100 ml for 10 g of wheat bran) and the contents were squeezed through a wet muslin cloth.

The enzyme extract was centrifuged at 10,000g for 30 min at 4C and the clear supernatant was used as the enzyme source.

2.5.Cellulase assay

1% carboxymethyl cellulose was used as substrates for assaying the activity of cellulase. The reaction mixture for cellulase assay contained 480 µl of CMC as substrate (prepared in glycine NaOH buffer of pH 9) and 20 µl of enzyme and was incubated at 55^{o}C for 10 min. The reaction was terminated by adding 1.5 ml of 3, 5- dinitrosalicylic acid reagent. Control for the enzyme assay was run simultaneously. The contents were boiled for 15 min and after cooling, the colour developed was read at 540 nm. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar (glucose for cellulase) per minute under the assay conditions (Miller 1959). All the experiments were carried out independently in triplicate and results presented are the mean of the three values.

2.6.Production of cellulase on various substrates

Erlenmeyer flasks (250 ml) containing 10 g each of various agricultural by-products/residues like wheat bran, gram bran, soya bran, maize bran, wheat straw, paddy straw and sugarcane bagasse were used as substrates for cellulase production. The substrates were moistened with mineral salt solution, autoclaved, inoculated and incubated. The enzyme was extracted and assayed.

2.7. Time course of enzyme production

The microorganism was grown in 10 g of wheat bran moistened with the mineral salt solution in 250-ml Erlenmeyer flasks. The enzyme was extracted and assayed at regular time intervals of 24 h for a week.

2.8. Effect of different moistening agents

Four mineral salt solutions prepared in distilled water were employed as moistening agents (MA) for wheat bran.
MA I (g/l): $MnSO_4.H_2O$, 0.5, K_2HPO_4, 1.5; pH8.0.
MA II (g/l): $MnSO_4.H_2O$, 0.5, $K_2HPO_4$1.5, yeast extract 2.0, peptone 2.0; pH 8.0.
MA III (g/l): Na_2HPO_4 11.0, Na_2HPO_4, 11.0, NaH_2PO_4, 6.0, KCl, 3.0, $MgSO_4$.7H2O, 0.1; pH 8.0.
MA IV (g/l): K_2HPO_4, 0.1, $(NH_4)H_2PO_4$, 1.0, MgSO.7H_2O, 0.5, $CaCl_2.2H_2O$, 0.1, $FeSO_4$, 0.1, MnSO4, 0.1; pH 8.0.
Besides these salt solutions, distilled water and deionized water were also used as moistening agents.

2.9. Effect of moisture level

The effect of moisture level on cellulase production was tested by varying the wheat bran-to-moisture ratio (w/v) in the range of 1:1.5–1:3.

2.10. Effect of temperature

Erlenmeyer flasks (250 ml) containing 10 g of wheat bran were inoculated and incubated at temperatures ranging from 37˚Cto50°C for 72 h.

2.11. Inoculum size

Wheat bran (10 g) after addition of 25 ml of deionized water in 250-ml Erlenmeyer flasks were autoclaved and inoculated with different amounts (5%, 10%, 15%, 20%) of 18-h-old culture and incubated at 37˚C for 72 h. The contents of the flasks were then harvested and assayed for enzyme activity.

2.12. Effect of additives

The effect of different additives was studied by supplementing the moistened wheat bran with glucose, fructose, malt extract, yeast extract, peptone, cottonseed cake and mustard oil cake at a concentration of 4% (w/w).

3. Purification

3.1. Ammonium Sulfate Precipitation

All purification steps were performed at 4°C. After 48 hr of cultivation at 37°C with shaking (250 rpm), the culture broth was centrifuged at 10,000g for 15 min. Enzyme in the cell-free supernatant portion of the culture was precipitated by addition of ammonium sulfate (NH4)2SO4 with constant stirring. Ammonium sulfate fractions of 0–40% and 40–80% (w/v) were collected by centrifugation at 10,000g for 30 min and the enzyme pellet obtained was dissolved in minimal volume of 50mM Tris-HCl (pH 8.0) buffer. The dissolved pellet was dialyzed against the same buffer with three changes. Dialysis was performed .

3.2. Ion-Exchange Chromatography

The enzyme extract (dialysate) was further purified using fast protein liquid chromatography 20mL of the dialysate was loaded on to a diethylaminoethyl (DEAE)-Sepharose column (1.5x20 cm). Before loading the sample, the column was preequilibrated with 50mM Tris-HCl buffer, pH 8.0. The column was washed with 2 column volumes of the same buffer and the adsorbed protein was eluted with a linear gradient of 0–0.8 M NaCl in equilibration buffer at a flow rate of 1mL/min. Each fraction of 3mL was collected for estimation of protein concentration (absorbance 280 nm) and CMCase activity (U/mL). The active fractions containing cellulose activities were pooled and stored at 4°C for further analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the active fractions was carried out to check the homogeneity of the enzyme and to determine its molecular weight. All the purification steps were performed at 4°C.

3.3. Analysis of Purification by SDS-PAGE

To check the purity of enzyme, SDS-PAGE was performed following the method of Laemmli (1970). The purified enzyme sample was mixed with 5x loading dye buffer in a ratio of 4:1. The sample mixture was subjected to heat denaturation for 5 min and centrifuged at 12,000 rpm for 1 min. The crude cellulase and column-purified cellulase from different steps of purification were loaded on 3 identical 10% acrylamide gels and the electrophoresis was carried out using 1x running buffer (200mM glycine, 0.1% SDS, 50mM Tris-HCl, pH 8.3) with a current of 2.5mA per lane. The first two gels were loaded with same samples of crude

supernatant and ammonium sulfate-purified cellulase but stained with silver staining and 0.25% (w/v) Coomassie brilliant blue (CBB) R-250, respectively. The third gel was loaded with column-purified cellulase fractions and visualized by silver staining protocol.

3.4. Molecular Mass

The molecular mass (*M*) of purified cellulase was determined using SDS-PAGE. Phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as markers.

4. Application

4.1. Biostoning of Denim fabric

Denim cloth was treated with the crude CMCase in a 250-ml Erlenmeyer flask with 10 steel balls (diameter 0.7 cm). At a solid:liquid ratio of 1:10, a 4×4-cm denim swatch, was immersed into 20 ml of 100 mM sodium phosphate buffer (pH 7.0) rotating at 200 rpm at 40°C for 1 h. The dosage of crude CMCase was 100-700 ECU per g of fabric. The amount of indigo dye released into solution was determined by assaying the absorbance at 370 nm. Denim dry weights were determined before and after enzymatic treatment.

5. Results and Discussion

Bacterial samples were isolated from the soil of which, best strains which produces better zone producing strain was chosen and preceded for further studies . Cellulase producing Bacteria were found commonly in all environments which enables them to degrade the cellulose found prevalent in waste materials. The Bacterial isolated in the present study was labeled as **sample 88** was found to be *Bacillus subtilis* subsp. inaquosorum ***strain*** KCTC 13429(T) (GenBank Accession Number: AMXN01000021) based on nucleotide homology and phylogenetic analysis. It was identified by IMTECH Chandigarh India and has been given National Centre of Biotechnology Information (NCBI) Accession no AMXN01000021 **. Different production parameters under solid state conditions were optimized.** Maximum cellulase production 71.15 IU/ml was obtained with wheat bran as carbon source in MA-1 mineral media, fructose and yeast extract as additives under 48 hr of production period at 37˚C, with 30% inoculum size. Moisture level of 1:2 (w/v) was found best for cellulase production. Increasing moisture level is believed to reduce the porosity of wheat bran, thus limiting oxygen transfer. At moisture levels below optimum, the enzyme titre was low which might be because of the decreased solubility of nutrients present in the solid substrate.

5.1 Purification

The purification of cellulase from cell-free supernatant was carried out by ammonium sulfate precipitation followed by ion-exchange chromatography using DEAE-Sepharose and Sephadex G -150.After 48 hr of incubation the broth was centrifuged at 10,000g and 4°C for 15 min. The cell-free supernatant containing crude enzyme had specific activity of 222.34IU/mg (Table 1). This was subjected to ammonium sulfate precipitation, and maximum cellulase activity observed was in the fraction precipitated at 80% saturation. After ammonium sulfate precipitation the enzyme gave specific activity of 333.3 IU/mg with 1.49 fold purification and 84.3% yield (Table 1). Ammonium sulfate precipitate enzyme was subjected to DEAE-Sepharose chromatography. Purification by ion exchange chromatography resulted in fractions from 19 to 32 of each 3-mL fraction size showing high protein content as analyzed spectrophotometrically (OD 280 nm) . These 14 fractions of 3mL each were pooled for estimation of protein concentration and CMCase activity . The fractions with higher CMCase activity were pooled for gel filteration Sephadex G 150 . The purified enzyme showed specific activity of 375 IU/mg with 1.68 fold and a final activity yield of 21.08 % (Table 1).

Table 1: Purification of Cellulase

Purification step	*Total volume(ml)*	*Total cellulase units(IU)*	*Protein(mg)*	*Specific activity (IU/mg)*	*Purification fold*	*% yield*
Crude enzyme	500	35575	160	222.34	1	100
0-80% Ammonium sulphate fractionation	500	30000	90	333.3	1.49	84.3
DEAE Sepharose	500	19000	56	339.2	1.52	53.4
Sephadex G150	500	7500	20	375	1.68	21.08

5.2 Molecular Characteristics of Purified Cellulase

The molecular mass (*M*) of purified cellulase was 32.5 kDa,determined by SDS-PAGE .

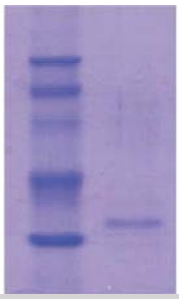

Figure SDS-PAGE of purified cellulase from *Bacillus subtilis subsp. inaquosorum* . protein markers [phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa)]; 1: purified cellulase.

5.3 Biostoning

The biostoning process aims at giving denim a more uniformly aged appearance. In fact, along with an external mechanical agitation, CMCase is mainly responsible for an effective indigo dye removal and weight loss from denim (27). Compared with only buffer treatment, crude CMCase significantly increased denim weight loss percent (table2) and indigo dye removal percent. This presents a more ecofriendly and efficient way than to use chemicals and stones for denim finishing.

Table 2: The result of biostoning denim fabric with crude CMCase and Control (Buffer only)

Preparation dosage (ECU/g)	Weight loss (%)	A370nm
Control	0.28±0.23 [a]	0.151±0.110 [b]
100	0.41±0.45 [a]	0.241±0.125 [b]
200	0.53±0.15 [a]	0.281±0.116 [b]
300	0.78±0.65 [a]	0.371±0.123 [b]
400	0.93±0.25 [a]	0.485±0.121 [b]
500	0.106±0.15 [a]	0.211±0.121 [b]
600	0.98±0.65 [a]	0.111±0.123 [b]
700	0.83±0.25 [a]	0.215±0.121 [b]

Data presented are the average obtained from three independent experiments with standard error(±SE)values. a:- $p<0.05$, b:- $p<0.01$

Analysis of color intensity on denim swatches was carried out using a HP Scanjet G4050 scanner. A circular area on the swatch, subjected to the combined enzyme and mechanical was scanned at 300 dpi resolution. The JPEG images obtained as a result of scanning were analyzed using Adobe Photoshop software. A histogram of color intensities was calculated for each sample using a blue channel of the software, and a percentile at level 32 (P32) was calculated. The difference between the average value of P32 obtained in the control (without cellulase) and the average value of P32 for the particular enzyme dosage was taken as an indicator of the abrasive activity of the enzyme, and it was expressed in relative units(table 3)

Table 3

Enzyme dosage	Relative Average value at P32
100	6
200	15
300	18
400	26
500	30
600	25
700	14

6. Conclusions

Cellulose is very resistant to hydrolysis due to its high degree of crystallinity. However, the isolation and characterization of novel cellulolytic strains from bacteria are now becoming widely exploited due to their rapid growth rate, enzyme complexity and extreme habitat variability. In the last decades, the high production cost and low yields of this enzyme are the major problems for industrial applications. Therefore, investigations on exploring different microbial strains for their potential to produce cellulase at economical level with enhanced productivity, is a major area of cellulase research. As such, this present study is an attempt to reveal the isolation, production, and potential applications of cellulase enzyme produced from ***Bacillus subtilis subsp. inaquosorum*** in denim industry.

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