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Optimization of *Bacillus cereus* MRK1 cellulase production and its Biostoning activity

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ABSTRACT

Cellulases are industrially important enzymes and they are produced by a variety of microorganisms. However, there are few studies on the production of these enzymes by Bacillus species there has been scarce reports on Bacillus cereus MRK1 isolated from paper sludge and its purification. Different intends such as incubation period, temperature, pH and effect of carbon and nitrogen sources were optimized for maximum yield of the enzyme. Initial optimization process showed pH 8, 32℃, xylan and yeast extract favoring enzyme production. The test strain showed its ability to secrete cellulase around 102 U/ml when it was grown in paper sludge supplemented medium. The purified cellulase recorded protein band around 60 kDa confirmed the presence of enzyme by zymogram. Purified cellulase showed its biostoning ability when applied on jean cloth.

Key words: Cellulases, *Bacillus cereus*, Xylan, Yeast extract, Paper sludge, Biostoning.

INTRODUCTION

Sludge is the largest by-product of the pulp and paper industry and disposal of sludge is a major solid waste problem for the industry **[1]**. It was predicted that a global shift in paper and paperboard production would result in the Asia-Pacific region emerging as a major producer of paper mill sludge [2, 3]. Paper sludge is a renewable, abundant and inexpensive resource for the bioconversion to biofuels and bioproducts. It is comprised of cellulose, a homologous polymer of glucose molecules connected by β-1, 4 linkages. Cellulase is an important commercial enzyme that widely used in food, animal feed, textile, pulp and paper, grain alcohol fermentation; starch processing, pharmaceuticals, malting and brewing industries [4]. Cellulase refers to a group of hydrolytic enzymes (endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidases) that are capable of hydrolyzing insoluble cellulose to glucose [5].

Several microorganisms including both bacteria and fungi have been found to produce a variety of cellulases for the degradation of cellulose. Fungal cellulases are produced in large amounts, which include all the components of a multi-enzyme system with different specificities and mode of action, i.e. endoglucanases, cellobiohydrolases (exoglucanases) and b-glucosidase, acting in synergism for complete hydrolysis of cellulose [6,7,8,9,10]. Bacterial cellulases have also been studied extensively [11, 12]. Among bacteria, *Bacillus* species produce a number of extracellular polysaccharide hydrolysing enzymes [13].

Cellulases from various sources have shown their distinctive features as they carry their specific pH optima, solubility and amino acid composition. Thermal stability and exact substrate specificity may also vary with the origin. The optimum pH generally lies between 4 and 5 and temperature is 40-50°C [14, 15]. CMCase activities alter with varying pH and temperature and characterization of produced enzyme require knowledge about optimum pH,

temperature stability and substrate specificity. Therefore, the enzyme is characterized to find out the best level of performance for enhanced efficiency of the process [14, 15]. Against all these backdrops, this study was aimed to investigate cellulase production from *Bacillus cereus* MRK1 in a paper sludge supplemented medium after the initial optimization parameters (pH, temperature, and carbon and nitrogen sources) and purified cellulase was applied as a biostoning agent in jean cloth.

MATERIALS AND METHODS

Sludge sample

Paper sludge samples were collected from Ashok Rai Paper and Pulp Industries, Chennai, Tamil Nadu. The samples were transferred to laboratory and stored at room temperature before use.

Isolation of bacterial strains

The collected samples were serially diluted up to 10^{-7} dilutions using sterile saline as a blank and the diluted samples were plated into sterile Nutrient agar (NA) using spread plate method. The NA plates were incubated at 37°C for 24 h. The isolated colonies were further purified by streak plate method using sterile media plates. The pure cultures were inoculated into sterile nutrient agar slants and nutrient broth for further use.

Screening for celluloytic strains

The isolated pure strains were screened for the production of extracellular cellulase using Carboxymethyl Cellulose (CMC) screening medium (0.5 g CMC; 0.1 g $NaNO_3$; 0.1 g K_2HPO_4; 0.1 g KCl; 0.05 g $MgSO_4$; 0.05 g yeast extract; 0.1 g glucose; 100 ml distilled water) [16]. The pure cultures were streaked at the center of the Sterile CMC agar plates and the plates were incubated at 37°C for 24 h. The observation was made to see the substrate utilized zone around the colony. Around 0.1% of congo red solution was over layered on the medium and kept for 15 min. Destaining was made using 1M NaCl to make the zone visible and clear. Only positive and better zone producing strain MRK1 was selected for further study.

Characterization and molecular identification of bacteria

The preliminary characterization of the isolated strain was done using Bergey's manual of systemic bacteriology [17]. The identity of the isolate was determined by sequence analysis of the 16S rDNA gene. The overnight cultured bacterial cells were lysed with lysozyme and the DNA was extracted by the phenol: chloroform (1:1) extraction method described by Ausubel *et al*. [18]. The 16S rDNA was amplified in PCR with the primer pair BcF (GGATTAAGAGCTTGCTCTTAT) and BtF (GATTGAGAGCTTGCTCTCAATA). The amplified region was then sequenced and subject to BLAST analysis for analyzing its phylogeny [19].

Inoculum preparation

The inoculum for further production of enzyme and other studies was prepared using Luria broth (LB) [20]. The pure culture was inoculated into sterile inoculum broth and was incubated at 37°C in a rotary shaker for overnight. The fresh over night culture was used as an inoculum for production of cellulase.

Cellulase production medium

Five hundred milliliters of sterile production broth was prepared in 1L conical flask and 5% inoculum was transferred aseptically in to the production medium (g/100ml: 0.01 MgSO4, 0.1 yeast extract, 0.2 KH_2PO_4, 0.7 K_2HPO_4, 0.05 Sodium citrate, 0.1 carboxymethyl cellulose, pH 7.0) [21]. The inoculated medium was incubated at 37°C for 48 h. The production medium was agitated at 200 rpm for better aeration and growth of the organism. Crude enzyme preparation was obtained from the supernatant after centrifugation of the broth culture at 5000g for 20mins and was examined for the total protein content and cellulase activity.

Cellulase assay

Cellulase activity was determined by estimating the reducing sugar produced during enzymatic reaction by dinitro salicylic acid (DNS) [22]. One milliliter of the enzyme solution was mixed with 0.5% of CMC and incubated at 37°C for 15 min to perform the enzyme substrate reaction. The contents were cooled and 1 ml of DNS was added and heated at 90°C for 5–15 min till slight reddish brown was developed. The contents were cooled and 1 ml of potassium sodium tartrate was added. The absorbency of the contents was measured at 540 nm against reaction mixture prepared using distilled water as blank.

Total protein content

The amount of protein was estimated by Bradford method [23] using Bovine Serum Albumin (BSA) as a standard according to the instruction manual of Quick Start Bradford Protein Assay.

Effect of incubation time

Around 500 ml of sterile production medium was prepared and 5% inoculum was added aseptically. The inoculated medium was incubated at 37°C with shaking around 200 rpm. After incubation, around 20 ml of culture was aseptically withdrawn periodically at 6 h intervals up to 72 h. The culture filtrate was examined for the total protein content and cellulase activity.

Effect of initial pH values and temperature

The production medium was adjusted at various levels of pH by NaOH solution (5, 6, 7, 8 9 and 10) and the effect of initial pH on cellulase production was studied. To study the effect of incubation temperature on cellulase production, the flasks with the production medium were inoculated and incubated at various temperatures (28ºC, 32ºC, 37ºC, 42ºC, 47ºC, and 52°C) for 48 h.

Effect of carbon source

Effect of various carbon sources namely glucose, xylose, xylan, sucrose, maltose and lactose on cellulase production was evaluated at 1%. The flasks were inoculated with 5% inoculum and incubated at 37ºC for 48 h.

Effect of nitrogen source

Effect of various organic and inorganic nitrogen sources namely yeast extract, casein, peptone, potassium nitrate and ammonium nitrate on cellulase production was evaluated at 1%. The flasks were inoculated with 5% inoculum and incubated at 37ºC for 48 h.

Paper sludge medium for cellulase production

After the initial optimization experiments, cellulase production was enhanced by using raw paper sludge as the production medium. The paper sludge sample at 1% (v/v) was used for cellulase production by replacing conventional carbon and nitrogen sources and trace elements. The paper sludge sample was sterilized and seeded with 5% inoculum and incubated at 37°C for 48 h. Aliquots of 10 ml of the culture filtrate were centrifuged at 5000 rpm for 15 min after the enzyme production. Cell free extract was stored at 4^{o}C and used for cellulase estimation and total protein content.

Purification of cellulase

The enzyme separation from the production medium was precipitated with 80% ammonium sulphate at 4°C for 24 h to precipitate all the proteins. Precipitate was separated by centrifugation around 10000 rpm for 10 min. The supernatant was discarded and the remaining precipitate was dissolved with 2 ml of 0.5 mM Tris-HCl buffer (pH 8). The partially purified enzyme was subjected to ion exchange chromatography with DEAE Sephadex A-50 column, 50 mM phosphate buffer (pH 7.0) as Running buffer and 1M NaCl in 50mM phosphate buffer as elution buffer (pH 7.0) for complete purification.

Molecular weight determination by SDS-PAGE

The molecular weight of the Purified cellulase was determined by sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out by the method of Laemmli et al. [24] with some modifications using 12% polyacrylamide gel. For the measurement of molecular mass of protein, commercial broad range molecular mass standard proteins were used. Protein bands were located by coomassie brilliant blue staining.

Cellulase zymogram

For the determination of cellulase activity, SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 40% for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. The renaturation of the enzyme proteins was carried out by placing the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mMβ-mercaptoethanol, and 1 mM EDTA at 4°C. The gel was then transferred onto a glass plate, sealed in film, and incubated at 37°C for 4-5 h. The gel was stained with 1% Congo red for 30 min, and destained in 1 M NaCl for 15 min. Clear bands indicated the presence of cellulase activity [25].

Application of purified cellulase as biostoning agent

Denim fabric was prewashed for 10 min at 60^{o}C with mild detergent. The fabric was cut into 5 × 5 cm (2 No's). Cellulase treatments were performed in 250 ml conical flasks containing 95 ml of 50 mM citrate buffer (pH 6.5) and magnetic pellet was added into conical flask to help the colour removal. In one conical flask, 5 ml of purified cellulase was added (test) and in another flask, 5 ml of distilled water which acted as control. The conical flasks were kept at 50^{o}C in a heating magnetic stirrer at 50 rpm for 30 min. After removing the fabrics from the conical flask, they were soaked for 10 min in 100 ml of 10 Mm NaOH. The fabrics were rubbed and then rinsed gently with

10 Mm NaOH for 2 min. Then it was finally rinsed with tap water. The fabrics were dried for 1 h at 105°C and air dried overnight at room temperature. The colour from both fabrics was observed and recorded.

RESULTS AND DISCUSSION

Sludges from pulp and paper mills are mainly cellulose fiber generated at the end of the pulping process prior to entering the paper machines. They are composed essentially of fibrous fines and some inorganics such as kaolin clay, calcium carbonate. Titanium dioxide and other chemicals used in the specific manufacturing process. The fibrous fines are 59-72% (dry basis) cellulose. 6-16% lignin and 7-10% hemicellulose [26]. Ash contents in the sludges range from 10 to 70%. Hence, it is pertinent to say that the organisms present in the sludge might have the ability to produce cellulase, an industrially important enzyme during the process of cellulose degradation.

In this study, the bacterial strains were isolated from the paper sludge obtained from Ashok Rai Paper and Pulp Industries, Chennai, Tamil Nadu. These samples usually contain the microbes doing spoilage of cellulose fiber substrates by producing cellulases. From the paper sludge sample, 12 bacterial strains were isolated which were later screened for cellulase production qualitatively. It was found that 4 strains showed positive results. Among 4 strains, an isolate 'MRK1' produced high cellulolytic zone and was used for further studies.

From microscopic appearance and the biochemical tests, the isolate was identified as *Bacillus* sp. and further confirmation was done by sequencing the 16S rDNA gene and compared with the GenBank databases using the BLASTN program. The sequence analysis of 16S rRNA gene for the isolated bacterial strain MRK1 shows the maximum homology with other *Bacillus cereus* from the database and has been submitted to the Genbank (JN575261).

The effect of incubation time on cellulase production was tested by incubating the production medium with the test isolate at different time intervals (6 h to 72 h). The growth of the organism is essential for the production of enzyme because most of the extra cellular enzymes are produced during log phase of the organisms. It was found that gradual increase in cellulase production occurred at 48 h after incubation (Fig. 1). Most of the *Bacillus* sp. are maintaining log phase from 3 h to 12 h of its growth. This variation of log phase timing is based on the nutrient present in the medium and the cultural condition of the organism [16].

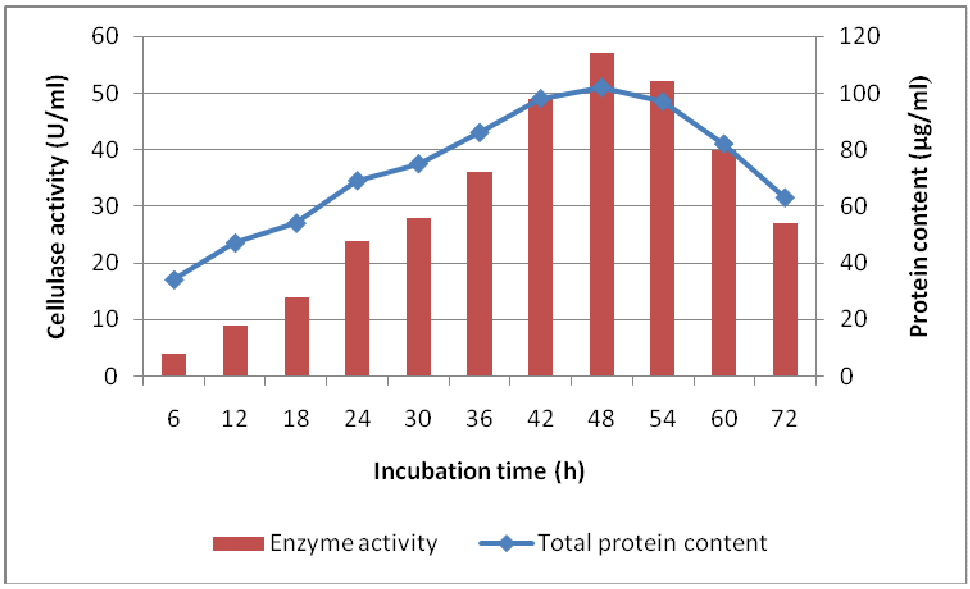

Fig 1: Effect of incubation time on cellulase production by *Bacillus cereus MRK1*

Hydrogen ion concentration of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. Maximum cellulase production was achieved at pH 8.0 by *Bacillus cereus* MRK1 (Fig. 2). The production of cellulase increased as pH of the medium increases and reaches maximum at pH 8 (63 U/ml). After pH 8.0 there was a decrease in enzyme production. Results suggest that there is a stimulation of enzyme production at alkaline pH. The obtained results coincide with Yang *et al.* [16] who reported that cellulase production was high between pH 7-9 for *Bacillus* spp.

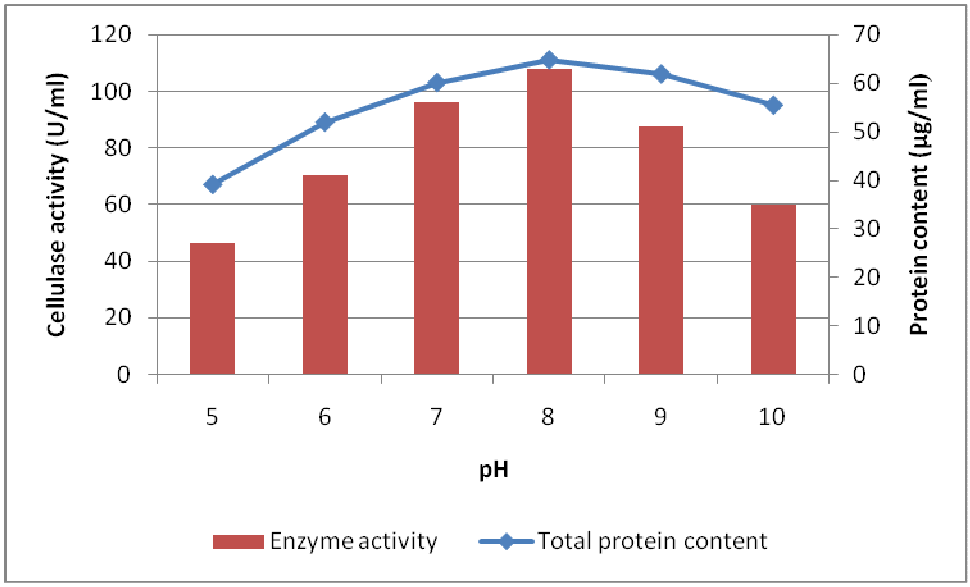

Fig 2: Effect of pH on cellulase production by Bacillus cereus MRK1

To study the effect of various temperatures on protein content and cellulase production, different temperature ranges (28ºC, 32ºC, 37ºC, 42ºC, 47ºC, and 52°C) were used in the study. It was found that like other mesophilic organisms, the higher cellulase activity was found (107 U/ml) at 32°C by the test isolate (Fig. 3). The temperature requirement of the organism is based on the nature of organisms. Many *Bacillus* spp. needed 32-37°C for better production of cellulase according to Yang et al. [16].

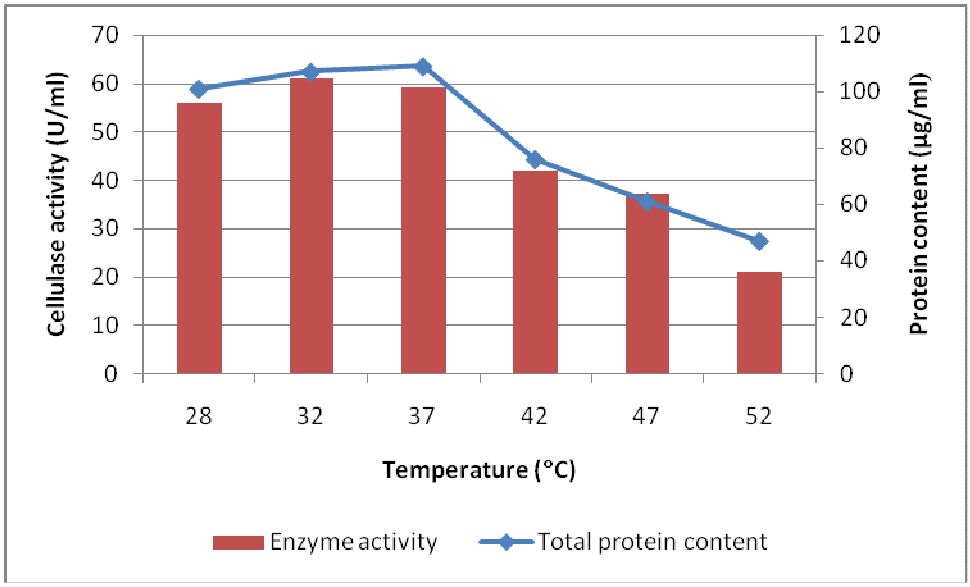

Fig 3: Effect of temperature on cellulase production by Bacillus cereus MRK1

Carbohydrates are soul energy source for most of heterotopic organisms and have great influence on enzyme production. High cellulase production was recorded by xylan (63 U/ml) and sucrose (57 U/ml) respectively (Fig. 4). Reduction in cellulase production was seen in glucose amended medium which might be due to feedback inhibition of enzyme production as the glucose is the end product [27].

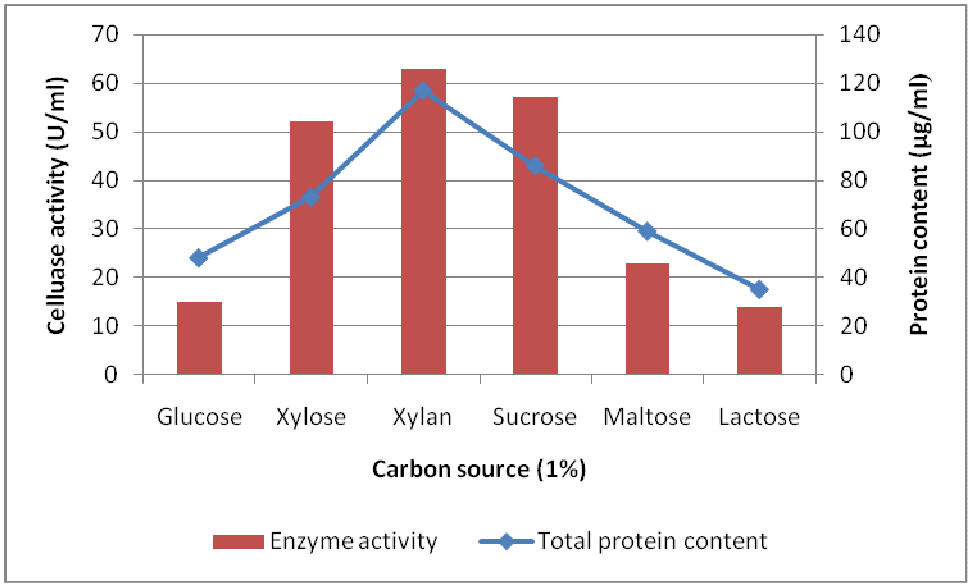

Fig 4: Effect of carbon source on cellulase production by Bacillus cereus MRK1

Different organic and inorganic nitrogen sources were evaluated for cellulase production by *Bacillus cereus* MRK1 Nitrogen sources are the secondary energy sources for the organisms which plays an important role in the growth of the organism and enzyme production. It was found that yeast extract favored cellulase production in case of organic nitrogen source where as ammonium nitrate was found to be more effective for the test strain in the inorganic nitrogen source category (Fig. 5).

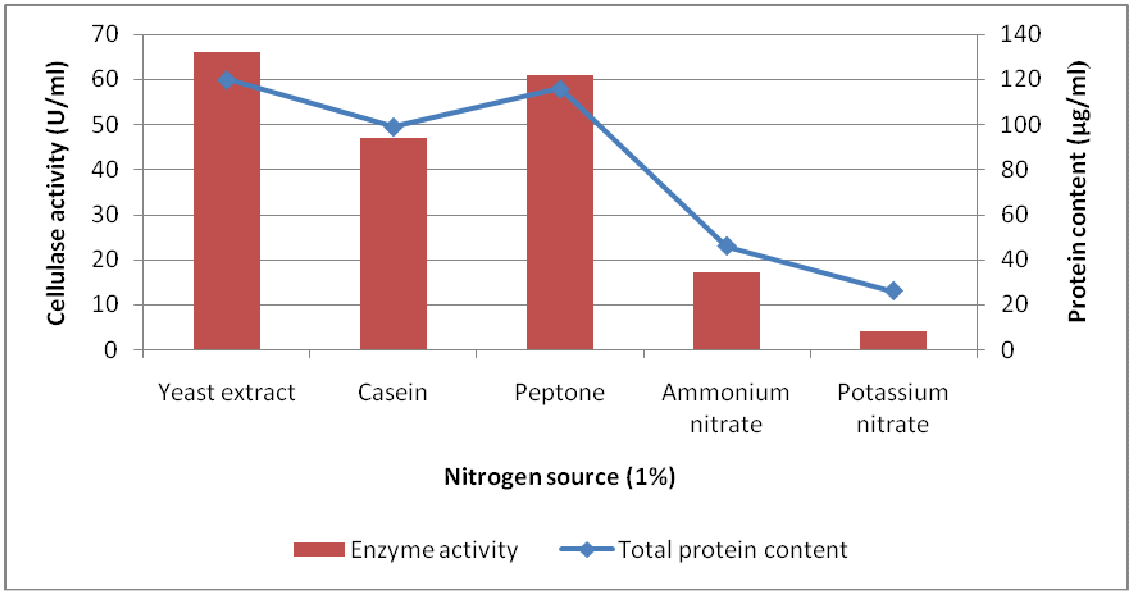

Fig 5: Effect of nitrogen source on cellulase production by Bacillus cereus MRK1

There was a considerable and high increase in the enzyme activity depicted by *Bacillus cereus* MRK1 when paper sludge was used as the primary substrate (Fig. 6). The test isolate recorded an enzyme activity of 66 U/ml after the initial optimization process. The enzyme activity rose to 102 U/ml in the sludge supplemented production medium. Thus, this optimized fermentation medium was further employed for the purification process.

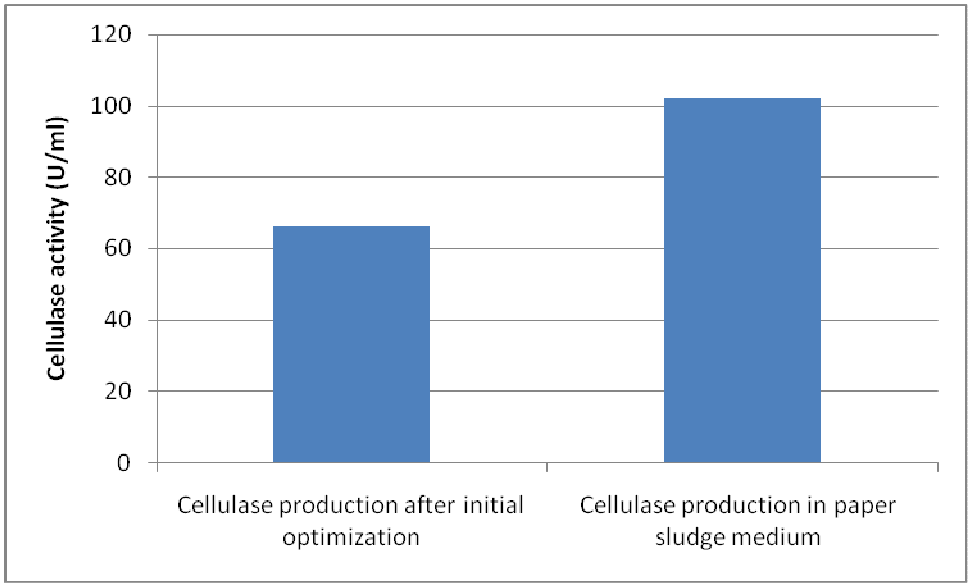

Fig 6: Cellulase activity recorded after initial optimization and in paper sludge medium

In enzyme fermentation process, the crude extracts contain different mixtures of proteins and undesirable products as organic acids and other metabolites. So that purification of the required favourable product must be take place by different purification methods. In most enzyme purification the precipitation of the crude total proteins after separating the culture from the fermentation broth by filtration or centrifugation and the culture supernatant is concentrated by salting out by solid ammonium sulphate as a precipitation agent [28]. In this study the optimized fermentation medium was precipitated using 80% ammonium sulphate and dialyzed. The purified enzyme was subjected to SDS-PAGE. It showed the presence of single band. The presence of protein band nearing the molecular weight 60 kDa confirmed the presence of enzyme. [25]. Cellulase zymogram revealed a clear yellow zone appearance in native gel showed the presence of cellulase.

It is difficult to control the amount of abrasion, the fabric was subjected to and acid-washing of jeans caused numerous environmental problems and a novel method of "stoning" jeans was needed to keep jeans smooth and comfortable. Against this backdrop, the impact of purified cellulase in biostoning was evaluated by the method of Arja Miettinen-Oinonen [29]. According to the observation the enzyme had the efficiency in removing the colour from denim fabric.

CONCLUSION

This study showed that a bacterial strain *Bacillus cereus* MRK1 has the ability to secrete cellulase to a greater extent. Initial optimization process showed pH 8, 32°C, xylan and yeast extract favoring enzyme production. The test strain showed its ability to secrete cellulase around 102 U/ml when it was grown in paper sludge supplemented medium. The purified cellulase recorded protein bands around 60 kDa confirmed the presence of enzyme. This study clearly indicates that cellulases may be used as a biostoning agent in textile industry since it possesses tremendous potential for making jeans smooth and comfortable via an environmental friendly technique.

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