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Cellulase Production by *Bacillus subtilis* isolated from Cow Dung

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ABSTRACT

Cellulases are the enzymes hydrolyzing cellulosic biomass and are produced by the microorganisms that grown over cellulosic matters. Bacterial cellulases possess more advantages when compared to the cellulases from other sources. Cellulase producing bacteria was isolated from Cow dung. The organism was identified using 16 SrDNA sequencing and BLAST search. Cellulase was produced and the culture conditions like temperature, pH, and Incubation time and medium components like Carbon sources, nitrogen sources and role of natural substrates were optimized. The enzyme was further purified using ethanol precipitation and chromatography. Cellulase was then characterized using SDS-PAGE analysis and Zymographic Studies. The application of Cellulase in Biostoning was then analyzed.

Keywords: Carboxy Methyl Cellulose, 16 SrDNA sequencing, Cow dung, Denim.

INTRODUCTION

Nowadays enormous amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Celluloses are regarded as the most important renewable resource for bioconversion. Many Cellulosic substances were hydrolyzed to simple sugars for making Single Cell Protein, sweeteners etc. It has been become the economic interest to develop an effective method to hydrolyze the cellulosic biomass.

Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters [1]. Increasing knowledge of mode of action of Cellulase; they were used in enzymatic hydrolysis of cellulosic substances [2]. Although a large number of

microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose *in vitro*. Numerous investigations have reported the degradation of cellulosic materials, but few studies have examined which microorganisms had met the industrial requirement. Fungi are the main cellulase-producing microorganisms, though a few bacteria have also been reported to yield cellulase activity.

Cellulases hold many potential industrial applications. In textile industries, they were used for the "Biopolishing" of fabrics for increasing its softness and brightness. They were also used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. It plays a major role in the conversion of renewable cellulosic biomass into commodity chemicals [3, 4].

Lee and Koo [1] showed that cellulase production was the most expensive step during ethanol production from cellulosic biomass, in that it accounted for approximately 40% of the total cost. The chance to obtain cheap ethanol will depend on the successful screening of novel cellulase producing strain. Since industrial bioconversions of lignocelluloses requires multifunctional cellulase with broader substrate utilization as well as the application of enzymes that can work efficiently in a wide range of temperatures and pH conditions used in the bioconversion of cellulosic material to bioethanol. The aim of this study was to isolate and identify new cellulase producing bacteria from Cow dung.

MATERIALS AND METHODS

Isolation and screening of Bacteria

The cow dung sample was collected from Adyar, Chennai and was serially diluted using sterile saline. The diluted samples were plated on Nutrient agar plates by spread plate method. The isolated colonies were further purified using streak plate technique and screened for Cellulase production. The screening was done by streaking the isolated colonies on screening medium (carboxymethylcellulose - 0.5g, $NaNO_3$ - 0.1g, K_2HPO_4 - 0.1g, KCl - 0.1 g, $MgSO_4$ - 0.05g, Yeast extract - 0.05g, Agar - 1.6 g, Glucose - 0.1 g, Distilled Water - 100ml) [5]. After 24 hours incubation the plates were flooded with 0.1% Congo red solution and left undisturbed for 15 minutes. To visualize clear zones formed by cellulase positive strains the plates were destained using 1M NaCl solution. Positive and better zone producing strain was chosen and continued for further studies.

Identification of Bacteria

Pure culture of the target Bacteria was grown overnight on Nutrient Broth for the isolation of DNA. The DNA was isolated from the bacteria using Cell Lysis method and 16S rDNA was amplified by Thermocycler (PTC – 100 TM Programmable Thermal Controller, USA) using the primers, Forward: 5' AGAGTTTGATCCTGGCTCAG 3', Reverse: 5' TACCTTGTTACGACTT 3' [6]. The amplified 16S rDNA PCR product was sequenced using automated sequencer (Chromus Biotech, Chennai). The Sequence Similarity Search was done for the 16S rDNA sequence using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast/). The unknown organism was identified using the maximum aligned sequence through BLAST search [7].

Production of Cellulase

Cellulase enzyme was produced using basal medium with following composition: 0.01% MgSO4, 0.1% yeast extract, 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.05% Sodium citrate, supplemented with 0.1% carboxymethyl cellulose (CMC) as carbon source [8]. Initial pH of the basal medium was adjusted to 7. Erlenmeyer's flask with 100 ml of autoclaved production medium inoculated with 1 ml of culture was incubated in rotary shaker at 200 rpm at 37°C for 72 hours.

Assay of Cellulase

The activity of Cellulase was assayed using DNS method ant total protein content by Bradford method. The bacterial crude was prepared by following method. 10 ml of culture was centrifuged at 5000 rpm for 15 minutes. The cell free extract was subjected to enzyme assay.

The DNS assay was carried out as follows. 0.2 ml of culture filtrate was mixed with 1% CMC in a test tube and incubated at 40°C for 30 minutes. The reaction was terminated by adding 3 ml of DNS reagent. The tube was then incubated at 100°C for 15 minutes followed by the addition of 1 ml of Rochelle salt solution. The OD was taken at 575 nm against blank. One unit of the cellulase activity refers to the amount of enzyme that released 1 µM of glucose [9]. Protein concentration was determined by Bradford method [10] using bovine serum albumin as standard.

Effect of Incubation time

Different incubation times (6, 12, 24, 48, 72, 96,120 hours) were employed to study their effect on the cellulase production. The culture filtrates were collected at respective time interval and assayed.

Effect of Temperature

The production was carried out at different temperatures such as 20, 25, 30, 35, 40, 45, 50°C to study their effect on cellulase production for 72 hours. The culture filtrates were then collected and assayed.

Effect of pH

The pH of the production medium was adjusted to 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 with 1N NaOH and 1N HCl. The production was carried out at 30°C to study their effect on enzyme production.

Effect of carbon sources

To identify the suitable carbon source for the Cellulase production, the carbon source (CMC) of the production medium was replaced with various carbon sources like Sucrose Mannose, Lactose, Glucose and starch. The assay was carried out after 72 hours of incubation.

Effect of Nitrogen sources

The production of cellulase was optimized by supplementing different nitrogen sources like gelatin, alanine, glycine, peptone, ammonium nitrate, ammonium chloride, ammonium sulphate. The nitrogen sources were added at 0.1% dry concentration in production medium.

Effect of Natural substrates

To identify the natural substrates facilitating better cellulase production, different natural substrates were used for the experimentation. The natural substrates include sugarcane baggase, filter paper, rice brawn, wheat brawn and coconut coir. The enzyme assay system was carried out after 72 hours of incubation.

Purification of enzyme

The culture fluid from the production media was collected and centrifuged. The culture supernatant was collected as crude enzyme extract for purification. To the culture supernatant, three volumes of 95% cold ethanol was added and the mixture was maintained in ice for 1 h with agitation. The precipitated crude extract was harvested by centrifugation and dissolved in 0.1 M Tris HCl buffer (pH 7.0). The precipitated enzyme was then 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 further purification [11].

Characterization of Protein

The further protein profile and the presence of purified Cellulase were confirmed by SDS PAGE analysis. The gel was stained using coomassie brilliant blue staining solution and destaining was done using alcohol acetic acid mixture solution [12].

Zymogram analysis

PAGE was executed according to the method of Laemmli [13]. Electrophoresis was done under non-denaturing conditions. Gels were incubated at 22°C in 50 mM NaCl (pH 7.3) and 2mM $CaCl_2$ with CMC as substrate. Cellulase release was detected by Congo red staining procedure**.**

Application-Biostoning of Denim fabric

Denim fabric was taken and prewashed with detergent for 10 minutes at 60ºC and was cut into two 5×5 size. The cellulase treatment was done in two conical flasks each containing 75 ml of 50 mM citrate buffer and the prewashed denim fabric. One was kept as a test in which 25 ml of culture filtrate was added. Another flask was assigned as a control in which 25 ml of distilled water was added. The conical flasks were kept on heated magnetic stirrer at 50 rpm for 30 minutes (50ºC). The fabrics were then soaked for 10 min in 100 ml of 10 mM NaOH and rinsed with 10 mM NaOH for 2 min followed by tap water. The fabrics were dried for one hour at 105ºC and air dried overnight at room temperature. The colour change occurred on both the fabrics were observed.

RESULTS AND DISCUSSION

Since most of the natural wastes were degraded by the native microbes present in it, the present study deals with the analyzing the microbes present in the cow dung for their ability of producing Cellulase. Around 21 bacterial samples were isolated from the cow dung of which, 9 strains were found to be Cellulase producer. Among them the better zone producing strain (Fig. 1) was chosen and preceded for further studies [14]. Cellulase can also be isolated from Cattle waste [15], woody biomass [16], Cow manure [17] and compost [18]. Cellulase producing Bacteria

were found commonly in all environments which enables them to degrade the cellulose found prevalent in waste materials.

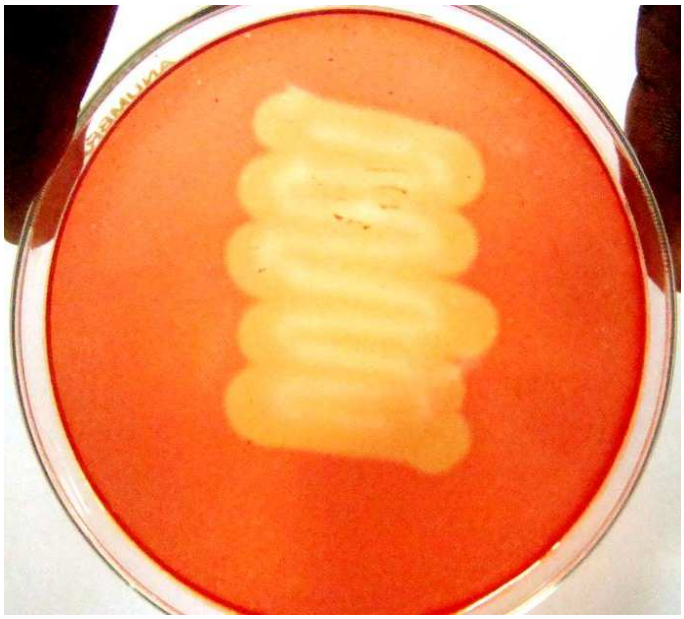

Fig. 1: Screening of cellulase producing bacteria

The better zone producing strain was assigned name as CEL PTK1 . The DNA from the strain CEL PTK1 was isolated and the 16S rDNA was amplified and sequenced. The BLAST analysis of the strain using its 16S rDNA sequence data showed that strain CEL PTK1 had highest homology (100 %) with *Bacillus subtilis*. When compared to morphological and biochemical characterization methods, 16S rDNA analysis is found to be the novel and accurate method for identifying unknown species. 16S rRNA sequencing appears to have the potential ability to differentiate strains at the subspecies level. Similarly, by 16S rDNA analysis, *Anoxybacillus flavithermus* and *Geobacillus thermodenitrificans* have been isolated by Abdelnasser and Ahmed in 2007 [7].

As the environmental parameters are essential for the production of Cellulase, they were optimized by shaken flask fermentation method [19]. There is a gradual increase in production occurred from 12th hour (Fig. 2) and maximum production was occurred at 72 hours with the enzyme activity of 30.33 U/ml. The Incubation time depends on the nutrients present in the medium and the cultural conditions of the organism [20].

The main parameters like temperature, pH are very essential parameters of the Cellulase production. To optimize the optimum temperature for the better Cellulase production, productions were made in various temperatures. The higher cellulase activity was found as 32.48 U/ml at 30°C for the Cellulase production (Fig. 3). The temperature requirement of the organism is based on the nature of organisms. A work done by Abdelnasser and Ahmed in 2007 [7], 75°C was found to be the optimum temperature for *Bacillus* sp.

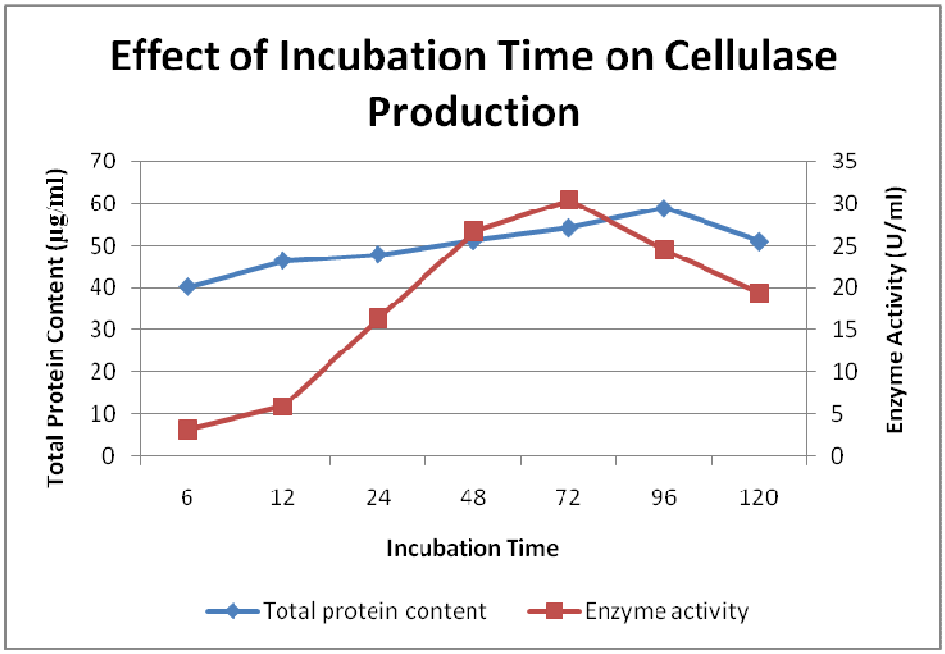

Fig.2 Enzyme production and total protein content of bacterial isolate at different incubation time

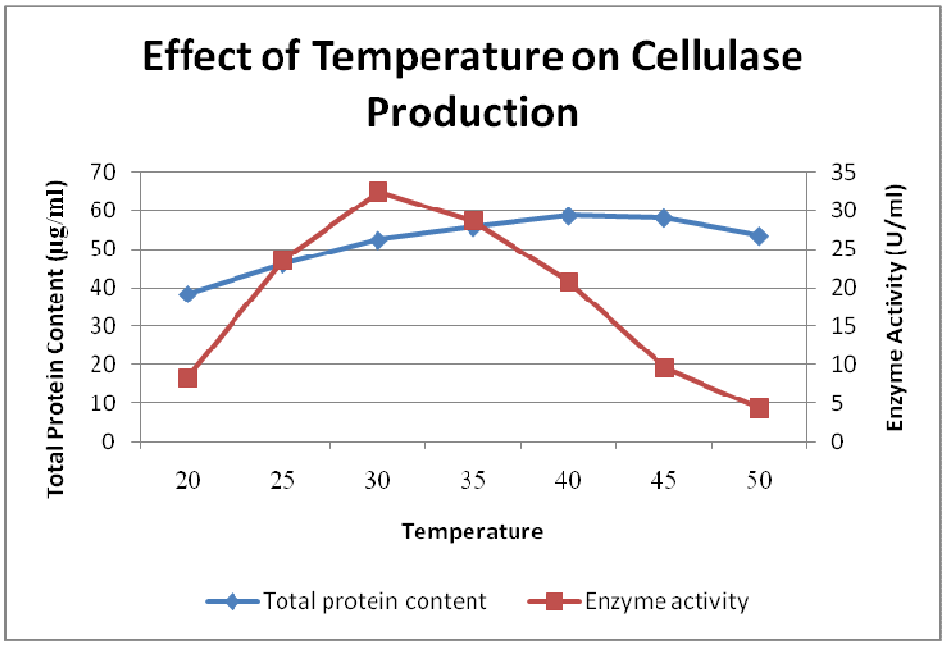

Fig.3 Enzyme production and total protein content of bacterial isolate at different Temperature

As the pH is found to be also impotent environmental parameter, varying pH were analyzed on Cellulase production. Maximum production of the enzyme (31.87 U/ml) was obtained at the pH 7.0 (Fig, 4). The pH of the selected organism was closely related to the optimum pH values of most of the *Bacillus* Spp. [21]. As an evident to the dependence of culture condition, *Bacillus subtilis KO* isolated from Egyptian soil possess optimal pH range from 6.5 to 7.0 [22].

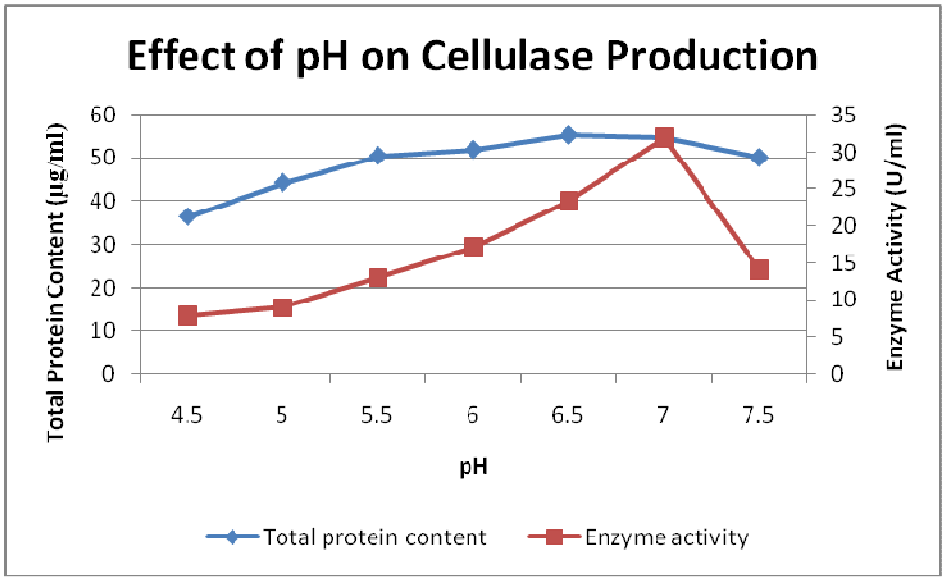

Fig.4 Enzyme production and total protein content of bacterial isolate at different pH

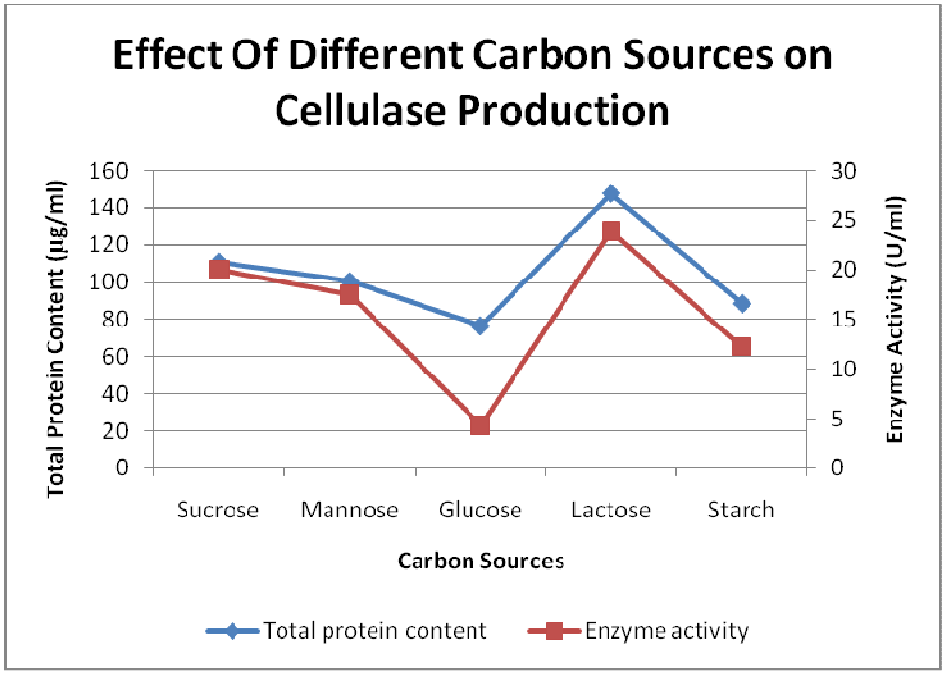

Fig.5 Enzyme production and total protein content of bacterial isolate at different Carbon source

Nutrient sources were found to be the next important factor for the Cellulase production. Since carbon is considered as the primary nutrient for the bacteria, different carbon sources like Fructose, Lactose, Maltose, mannitol, sucrose and starch were analyzed for the cellulase production. Maximum production of Cellulase of 23.96 U/ml was observed when lactose was served as the carbon source (Fig. 5). Hence, lactose was served as the better carbon source for

the Cellulase production. In a similar work done by Teodoro et al [23], Maltose has been reported as the best nitrogen source for *Bacillus* Sp.

Next to the carbon, Nitrogen was served as important nutrient source for the Cellulase production. Hence, different nitrogen sources like (Yeast extract, casein, gelatin, peptone, beef extract, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate) were applied as nitrogen sources for the Cellulase production. Peptone (Fig. 6) is found to be the better nitrogen source as it increases the production of Cellulase up to 29.63 U/ml. Earlier in a study, Starch was found to be the best carbon source. But, the source may vary depending on the strain and culture conditions [24]. For example, the similar *Bacillus subtilis* strain isolated from earthworm gut shows better production of Cellulases when Malt extract as Nitrogen source [25].

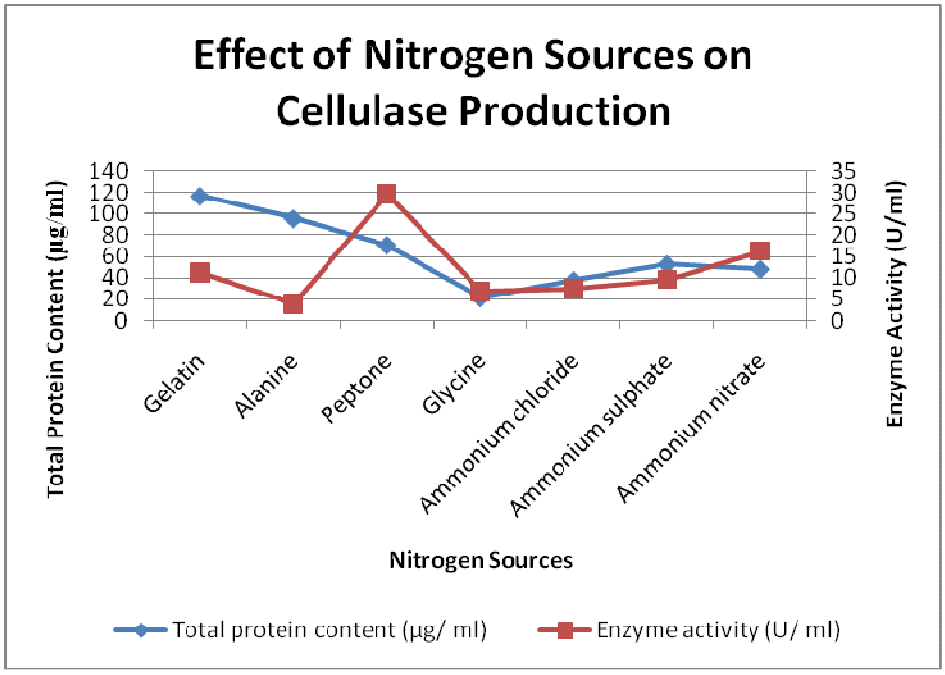

Fig.6 Enzyme production and total protein content of bacterial isolate at different Nitrogen source

The real and beneficial production of enzyme is produced from the natural sources and industrial wastes. In this study, several natural and industrial wastes were used as substrates. The results (Fig. 7) revealed that the bacterial isolate has produced maximum quantity of cellulase from Wheat bran (20.96 U/ml). The pattern of the substrate specificity for the production by our organism is similar to that of produced by *Bacillus amyoliquefaciens* [26].

The commonly used animal feeds like sugarcane bagasse, sugar beet pulp/husk, orange bagasse, oil cakes, apple pomace, grape juice, grape seed, coffee husk, wheat bran, cereals, straw, leaves, corncobs were disposed in environments [27]. They were degraded by bacteria and fungi. So, those waste materials can be used as substrates for the cellulase production [28].

The Cellulase enzyme produced was extracted and purified using ethanol precipitation and chromatography [12]. Electrophoretic analysis of extracellular Cellulase from the isolate has

been carried out. SDS-PAGE results showed the presence of multiple bands since along with cellulase some other proteins can be produced by the organisms. But the Presence of protein band nearing the molecular weight 60 Kda confirms the presence of enzyme. Molecular weight of Cellulases isolated from *Bacillus* Sp. was identified using SDS-PAGE analysis [29].

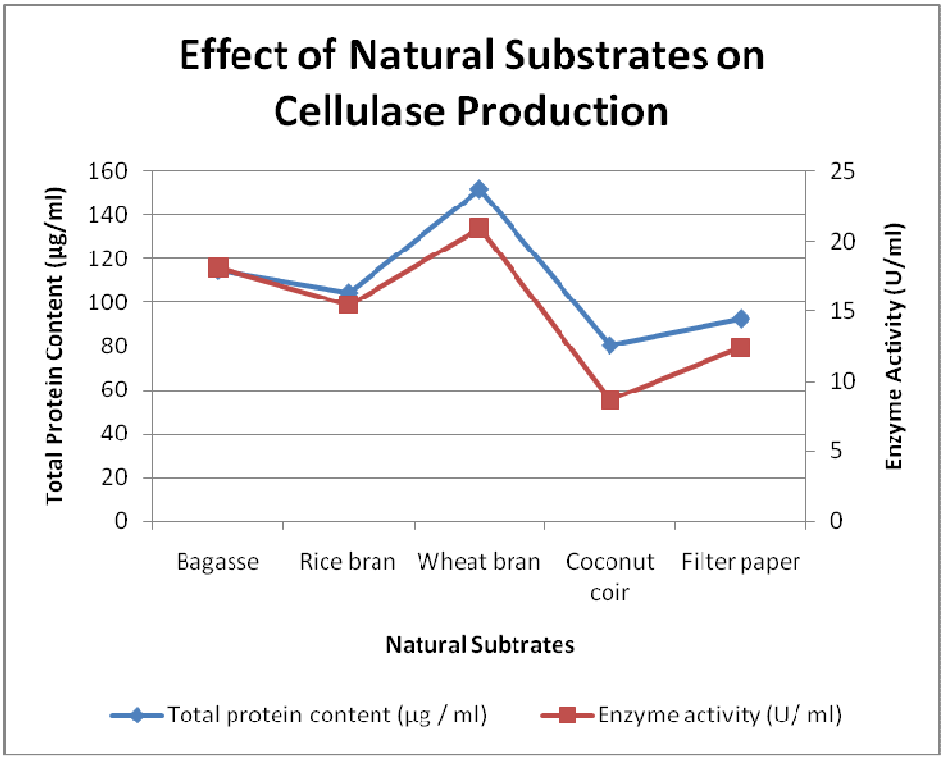

Fig.7 Enzyme production and total protein content of bacterial isolate at different Natural substrates

The zymogram of the present study shows the presence of Cellulase as yellow band due to staining with Congo red solution. In 1999, Choi and Kim [30] purified Cellulase was visualized by SDS-PAGE and zymogram analysis using Triton-X-100. The enzymes produced by *Bacillus* Sp. Can be detected using zymographic techniques are widely used [31, 32].

Since acid washed jeans are causing environmental pollution, an alternative stoning method was needed to keep our jeans comfortable and to prevent environmental pollution. Arja Miettinen-Oinonen [33] was the one who revealed that cellulases can be used as Biostoning agent. The main objective of Biostoning is to give a uniformed aged look to the Denim Fabric [34]. The endo-action of the cellulases are important for the removal of indigo dye from the denim fabric [35]. The observation revealed that the purified enzyme was effective in removing the stain from the fabric. But stone washing after enzyme treatment is needed for best result. Nowadays Biostoning works as same as traditional stone washing but without causing damage to the fabric [36].

CONCLUSION

From the results it has been revealed that the Cow dung is served as the good isolation source for the Cellulase producing microorganism as it is rich in cellulose. It is also found that cellulases are not only served as the cellulosic enzyme and also having applications on various industries including jeans processing. Such cellulase enzyme producing *Bacillus subtilis* strain (CEL PTK1) was isolated from cow dung. The isolated organism was identified by 16S rDNA sequencing and the optimal medium and environmental conditions were identified. The application of cellulase on biostoning of denim fabric was also evidenced.

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