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Cellulase Production by *Bacillus pumilus* EWBCM1 under Varying Cultural Conditions

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Abstract: A *Bacillus pumilus* EWBCM1 isolated from earthworm gut (*Eudrilus eugeniae*) was tested for its abilities to hydrolyze the structural polysaccharides. The effect of different production parameters such as pH, temperature, carbon source, nitrogen source (Organic and Inorganic), NaCl concentration, surfactants, metal ions, inoculum size and incubation time on cellulase production by the isolated bacterial strain were studied. The enzyme production was assayed in submerged fermentation (SmF). Maximum cellulase activity was found at pH 6, 37°C, galactose, malt extract, ammonium molybdate, calcium chloride, 2.5% NaCl, Tween-20, 72 hrs, 2% inoculum. A higher titer of cellulase enzyme activity (0.5851±0.006 IU/ml) was obtained in the optimized production medium.

Key words: Cellulose · CMCase · Earthworm · *Eudrilus eugeniae* (Kinberg) · Mid-gut · SmF

INTRODUCTION

Cellulose is probably the most abundant biological compound on terrestrial and aquatic ecosystem. It is the dominant waste material from agricultural industry in the form of leaves, stalks, stems and husk and is one of the most abundant renewable resources. By means of chemical or bioconversion methods, it is possible to transform this insoluble polymer into glucose, an excellent substrate for industrial fermentation. Mainly bacteria, fungi and actinomycetes achieve bioconversion of these materials. Cellulose the largest component of plant residues enters terrestrial ecosystems [1] and therefore represents a huge source of energy for microorganisms, the main agents responsible for soil organic matter decomposition [2].

In nature, cellulolysis occurs as a result of the combined action of fungi and bacteria with different substrate requirements that shift their biomasses depending on what substrate is being metabolized [3]. The type of microorganisms involved depends on the environmental conditions; under aerobic conditions, they are mainly fungi, actinomycetes and bacteria and under anaerobic conditions, they are almost exclusively bacteria [4]. Cellulase is an enzyme used for the conversion of lignocellulosic residues and used for the production of ethanol, single cell protein, bleaching of pulp, for treatment of waste papers and for fruit juice extraction. Cellulolytic activity is a multi-complex enzyme system and consists of three major components; endo-β-glucanase (EC 3.2.1.4), exo- β-glucanase (EC 3.2.1.91) and β –glucosidase (EC 3.2.1.21).

The aim of this work was to optimize the cultural condition for the production of cellulase enzyme by a *Bacillus pumilus* EWBCM1 isolated from earthworm gut *Eudrilus eugeniae* (Kinberg).

MATERIALS AND METHODS

Effect of pH on Cellulase Production: The effect of optimum pH for cellulase production by the experimental microorganisms was determined by culturing the bacteria in the production media with different pH. The experiment was carried out individually at various pH such as 3, 4, 5, 6, 7, 8 and 9. The enzyme assay was carried out individually after 72hours of incubation at 37°C.

Effect of Temperature on Cellulase Production: Temperature plays an important role for the production of extracellular cellulase by the test organism. The effect of temperature on cellulase production was studied by incubating the culture media at various temperatures such as 10, 20, 30, 40, 50 and 60 along with arbitrary control at 37°C.

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Effect of Carbon Sources on Cellulase Production: To identify suitable carbon source for the cellulase production by the test organism, the following carbon sources were tested. The production medium containing Carboxymethylcellulose, act as a carbon source. This CMC was replaced by glucose, mannitol, cellulose, sucrose, rhamnose, mannose, raffinose, trehalose, sorbitol, galactose and starch. These carbon sources were tested individually at the concentration of 1% with dry substrate in the optimized production medium. The enzyme assay was carried out after 72 hours of incubation at 37°C.

Effect of Organic Nitrogen Sources on Cellulase Production: The cellulase production by the selected bacterium was optimized by supplementing different organic nitrogen sources. For this, seven different organic nitrogen sources were tested individually at the concentration of 0.5% with dry substrate in the optimized carbon sources in production medium. The organic nitrogen sources used were yeast extract, glycine, peptone, gelatin, urea, casein and malt extract. The organic nitrogen source that results maximum cellulase production was then used for further study.

Effect of Inorganic Nitrogen Sources on Cellulase Production: The cellulase production by the selected bacterium was also optimized by supplementing different inorganic nitrogen sources. The different inorganic nitrogen sources used for the cellulase production were ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium molybdate and potassium nitrate. They were tested individually at the concentration of 0.5% in production medium. The inorganic nitrogen source that results maximum cellulase production was then used for further study.

Effect of Metal Ions on Cellulase Production: Normally, the production medium contains magnesium sulphate, sodium nitrate, potassium chloride, ferric sulphate and di potassium hydrogen phosphate. In the present study to enhance cellulase production aluminium sulphate, ferric chloride, zinc sulphate, nickel sulphate, copper sulphate, manganese sulphate, mercuric sulphate and calcium chloride were tested as the source of metal ions. In this study they were incorporated individually into the production medium at the concentration of 0.02%. The effect was determined after 72hours of incubation.

Effect of NaCl on Cellulase Production: The effect of NaCl on cellulase production was studied by supplying various concentration of NaCl to the production medium. The experiment was carried out individually at various concentration of NaCl such as 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0%.

Effect of Surfactants on Cellulase Production: To identify the surfactants facilitating cellulase production, four different surfactants were used for experimentation. They were Tween-20, Tween-80, SDS (Sodium Dodecyl Sulphate) and PEG (Poly Ethylene Glycol). The selected surfactants were tested individually at the concentration of 0.2% in the optimized production medium.

Effect of Incubation Time on Cellulase Production: The effect of incubation on cellulase production by the experimental microorganisms was determined by culturing the bacteria in the production media. The experiment was carried out individually at various incubation times. They were 24, 48, 72, 96 and 120hours. The enzyme assay system was carried out individually after 72 hours of incubation.

Effect of Different Concentration of Inoculum Level on Cellulase Production: Different concentration of inoculum level such as 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4% were tested for their ability to induce cellulase production in the production medium.

RESULTS

Effect of pH on Cellulase Production: Cellulase production by the *Bacillus pumilus* EWBCM1 isolated from earthworm mid-gut was adjusted under varying cultural conditions. The cellulase production after 72 hours of incubation at 37°C under various pH (Fig. 1). Maximum cellulase production was recorded at pH 6.0 (0.2530±0.008 IU/ml). The minimum cellulase production was recorded at pH 3 (0.007±0.006 IU/ml).

Effect of Temperature on Cellulase Production: Among the various temperatures tested, the maximum cellulase production was obtained at 37°C temperature (0.2740±0.006 IU/ml), followed by this, at 40°C temperature (0.2407±0.006 IU/ml) was the second best temperature on cellulase production. On the other hand, the minimum amount of cellulase production was observed at temperature 60°C (0.0296±0.006 IU/ml) (Fig. 2).

Effect of Carbon Sources on Cellulase Production: The effect of carbon sources on cellulase production by the candidate species after 72 hours of incubation period at 37°C is given in fig. 3. Here the maximum cellulase production was recorded in galactose (0.5851±0.006 IU/ml)

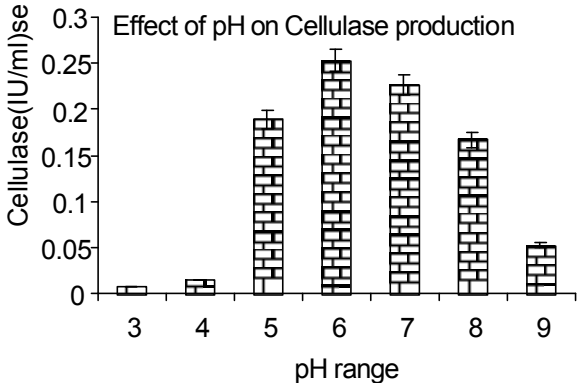

Fig. 1: Effect of pH on cellulase production

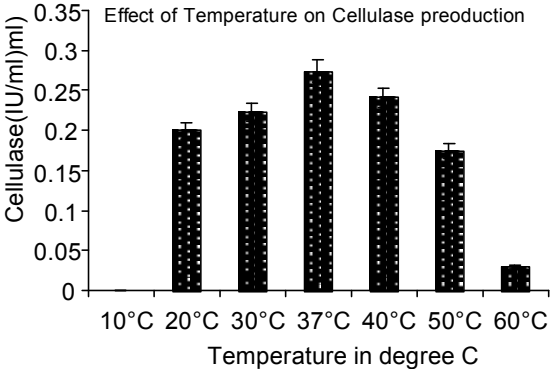

Fig. 2: Effect of temperature on cellulase production

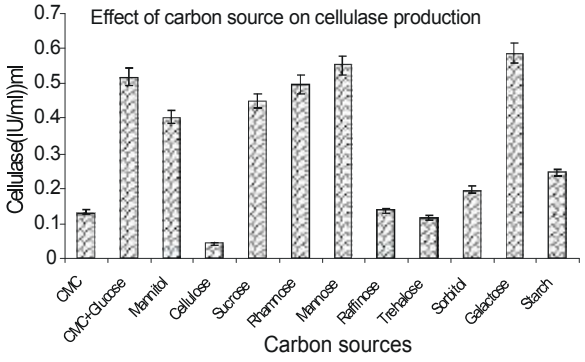

Fig. 3: Effect of carbon source on cellulase production

supplemented medium. The minimum cellulase production was recorded in cellulose (0.0419±0.004 IU/ml) added medium.

Effect of Organic Nitrogen Sources on Cellulase Production: The effect of different kinds of organic nitrogen sources on cellulase production after 72 hours of incubation period at 37°C showed maximum amount of enzyme production in malt extract (0.5666±0.002 IU/ml) supplemented medium and minimum amount of cellulase production in urea and glycine (0.0851±0.006 IU/ml) supplemented medium (Fig. 4).

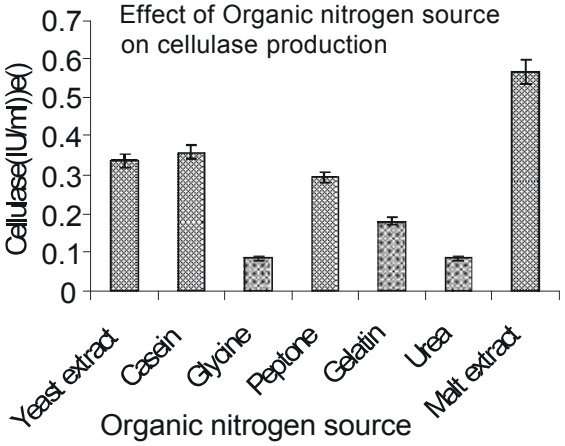

Fig. 4: Effect of organic nitrogen source on cellulase production

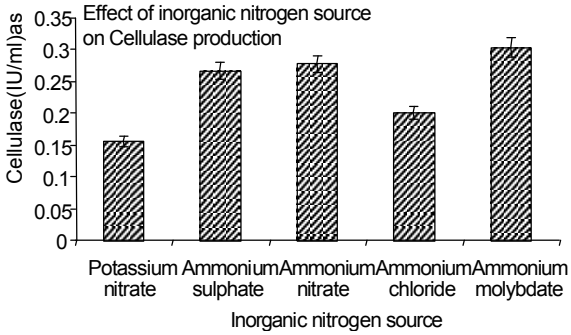

Fig. 5: Effect of inorganic nitrogen source on cellulase production

Effect of Inorganic Nitrogen Sources on Cellulase Production: The effect of different kinds of inorganic nitrogen sources on cellulase production after 72 hours of incubation period at 37°C showed maximum amount of enzyme production in ammonium molybdate (0.3036±0.006 IU/ml) supplemented medium and minimum amount of cellulase production in potassium nitrate (0.1555±0.006 IU/ml) supplemented medium (Fig. 5).

Effect of Metal Ions on Cellulase Production: Among the tested metal ions, the maximum amount of enzyme production was recorded in calcium chloride (0.1851±0.006 IU/ml) added medium. Followed by this, aluminium sulphate (0.1555±0.006 IU/ml) was the second best metal ions on cellulase production, whereas the minimum amount of cellulase production was observed in nickel sulphate (0.0148±0.006 IU/ml) supplemented medium (Fig. 6).

Effect of Different Concentration of NaCl on Cellulase Production: The effect of various concentration of sodium chloride was tested on cellulase production

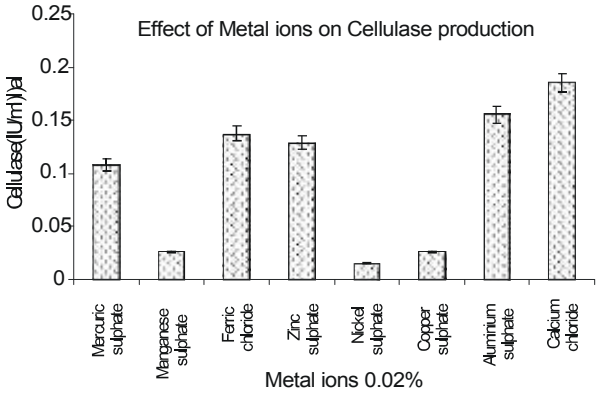

Fig. 6: Effect of metal ions on cellulase production

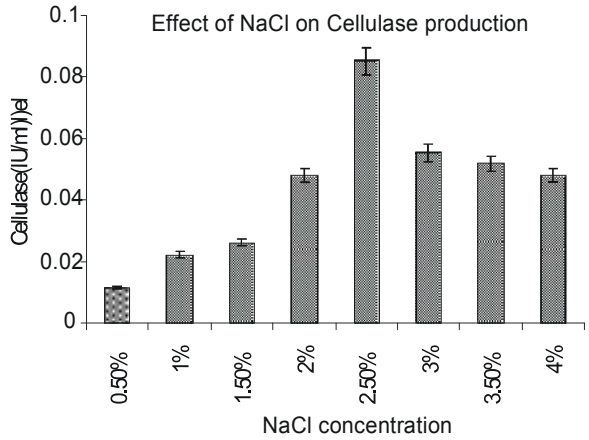

Fig. 7: Effect of NaCl on cellulase production

after 72 hours of incubation period at 37°C. Among the tested concentration, the maximum amount of enzyme production was observed in 2.5% sodium chloride (0.0851±0.006 IU/ml) supplemented medium. The lowest amount of enzyme production was recorded in 0.5% NaCl (0.0111±0.002 IU/ml) supplemented medium (Fig. 7).

Effect of Surfactants on Cellulase Production: The effect of different kinds of surfactants was tested on cellulase production after 72 hours of incubation period at 37°C. Among the tested surfactants, the maximum amount of enzyme production was recorded in Tween-20 (0.2222±0.006 IU/ml) added medium. The minimum amount of cellulase enzyme production was recorded in SDS (0.0148±0.006 IU/ml) supplemented medium (Fig. 8).

Effect of Various Incubation Intervals on Cellulase Production: The effect of different kinds of incubation time was tested on cellulase production. The maximum amount of cellulase production was observed in 72hours incubation time (0.5740±0.002IU/ml). The minimum amount of cellulase production was obtained in 120hours of incubation time (0.0653±0.002IU IU/ml) (Fig. 9).

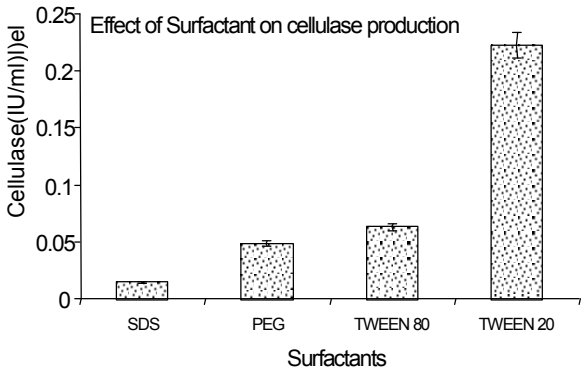

Fig. 8: Effect of surfactant on cellulase production

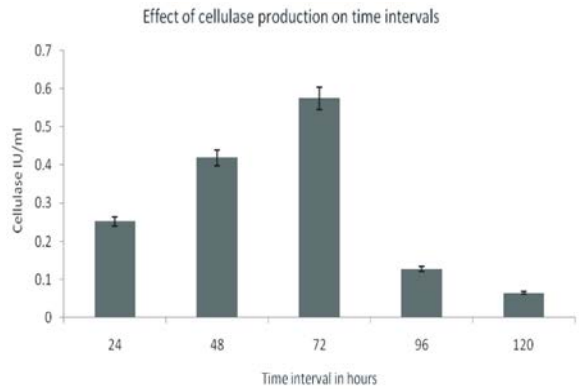

Fig. 9: Effect of various incubation time intervals on cellulase production

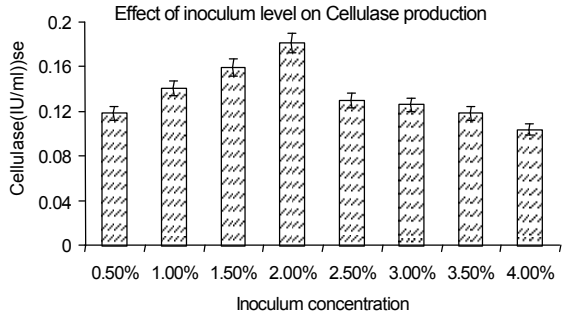

Fig. 10: Effect of inoculam level on cellulase production

Effect of Different Concentration of Inoculum on Cellulase Production: The initial inoculum level in the CMC media is a critical factor in fermentation process. In the present study, it has played an important role in cellulase production with sugarcane bagasse by *Bacillus sp*. The maximum cellulase specific activity was registered at the 2% (0.1814±0.0 IU/ml) of inoculum level. On the other hand, the minimum amount of cellulase production was observed at 4% of (0.1037±0.006 IU/ml) inoculums level (Fig. 10).

DISCUSSION

Several studies have been carried out to produce cellulolytic enzymes in biowaste degradation process by several fungi such as *Trichoderma sp*., *Penicillium sp*. and *Aspergillus sp* [5]. Similarly cellulolytic property of bacterial species like *Pseudomonas*, *Cellulomonas*, *Cellulovibrio* and *Sporocytophaga sp*. was also reported [6]. The cellulase system of the mesophilic cellulolytic aerobe, *Cellulomonas fimi* is one of the first studied and has since been one of the most studied bacterial cellulase systems. The specific cellulolytic activity shown by the bacterial species was reported to depend on the source of occurrence.

Many spore forming bacteria have been isolated from various sources such as feed stock from cattle waste [7], cow manure [8], woody biomass [9], municipal solid waste. Similarly many strains of cellulolytic anaerobic bacteria have been reported from various sources as human colon [10], estuarine sediments [11], fresh water sediments [12] and decomposing vegetation [13]. The common occurrence of these bacteria in various natural environments enables them to be responsible for the degradation of cellulose that occurs in various amount of biowaste.

Solomon *et al*. [14] achieved hydrolysis of saw dust using cellulase with activity of 0.0561 IU/ml. Some features of natural cellulosic materials are known to inhibit their degradation/bioconversion [15]. These are degree of crystallinity and lignifications and the capillary structure of cellulose. The crystallinity and lignifications limit the accessibility and susceptibility of cellulose to cellulolytic enzymes and other hydrolytic agents [16]. However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported [17]. Pretreatment of cellulose opens up the structure and removes secondary interaction between glucose chains [18].

Solomon *et al*. [15] produced cellulase of 0.056425 IU/ml from the growth of *Aspergillus flavus* on bagasse pre-treated with using ball milling and caustic soda. *A. flavus* grown on sawdust gave the highest cellulase activity of 0.0743 IU/ml, while bagasse and corncob gave 0.0573 IU/ml and 0.0502 IU/ml, respectively [19]. The depression in cellulase activity between 20^{th} and 30^{th} hr, common to all three substrates, may be due to cumulative effect of cellobiose, a dimer of glucose which is known to inhibit both endoglucanase and β-glucosidase [20]. Hatakka [21] also suggested that delignification produces aromatic water-soluble products which can repress the cellulolytic action of enzyme. The highest cellulase productivity with sugarcane may be due to its very high percentage of cellulose which is the major component of cell walls.

The production of cellulase (0.3 U/ml) by the wild type *Bacillus pumilus* in 24h of growth at optimum pH 6.5, optimum temperature 50°C and Ca^{2+} [22]. In recent years, some important agro-industrial residues such as sugarcane bagasse, sugar beet pulp/husk, orange bagasse, oil cakes, apple pomace, grape juice, grape seed, coffee husk, wheat bran, cereals, straw, leaves, corncobs etc. have been used as substrates. In most parts of the country, these materials are mainly used as animal feeds. A large quantity is left on farmlands to be decomposed by microorganisms such as bacteria and fungi [23, 24].

The type of strain, culture conditions, nature of the substrate and availability of nutrients are the other important factors affecting yield of enzyme production [25]. It is crucial to provide optimized water content and control the water activity for good enzyme production. Agro-industrial substrates are considered best for enzyme production. However, some newly developed agro-industrial wastes used for cellulase production are banana wastes, rice straw, corncob residue, rice husk, wheat straw, banana fruit stalk and coconut coir pith.

It was concluded that from economic point of view *Bacillus pumilus* EWBCM1 was optimized in various production parameters like pH, temperature, carbon source, nitrogen source (Organic and Inorganic), NaCl concentration, surfactants, metal ions, inoculum size and incubation time. So it can be used for cellulase production on cheaper and more easily available resources than on expensive and refined media.

CONCLUSION

The data gathered in this study provides evidence for the cellulase producing ability of the earthworm gut bacterial isolates. The production of cellulase and cellulase-lignocellulosic substrate interactions of bacterial strains in the earthworm gut was the evident through this study. More over this study gives us a hint as well as the microbial wealth of cellulase producing bacteria which can be harnessed for biotechnological processes.

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