Optimization of Cellulase Production of *Pseudomonas Aeruginosa* Sg21 Isolated From Sacred Groove, Puducherry, India

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Abstract- The efficient cellulase producing bacteria was isolated from the soil of sacred groove and identified as *P. aeruginosa* SG21 by 16S rRNA sequencing. The objective of this study is to perform biochemical testing, antibiotic sensitivity testing and optimizing the growth conditions of the bacteria *P. aeruginosa* SG21. The culture conditions like pH, temperature, carbon sources, and nitrogen sources were optimized. The optimum conditions found for cellulase production are 35 and 40°C at pH 6-10 with CMC as carbon source and urea as nitrogen source.

Index Terms- cellulase, optimization, P. aeruginosa

I. INTRODUCTION

Cellulose is an important structural component of the primary cell wall of green plants and many forms of algae . Some species of bacteria secrete it to form biofilms (Romeo 2008). Cellulose is the most abundant organic polymer on Earth (Klemm, 2005). The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest (Bhat, 2000). It is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable Bioresource produced in the biosphere (100 billion dry tons/year) (Zhang and Lynd, 2004). Approximately 70% of plant biomass is locked up in 5- and 6-carbon sugars (D- xylose, D- arabinose, D- glucose, D-galactose, D-mannose) which are found in lignocellulosic biomass comprised of mainly cellulose, lesser hemicelluloses and least of all lignin (Sadhu and Maiti, 2013). At the molecular level, cellulose is a linear polymer of glucose composed of anhydroglucose units coupled to each other by β-1, 4 glucosidic bonds. The number of glucose units varies from 250 to 10,000 depending upon the source. The nature of cellulosic substrate and its physical state are important in its bioconversion.

Biodegradation of cellulosic waste is accomplished by a concerted action of several enzymes, the most predominant of which are the cellulases- produced by a number of microbial species and comprise several different enzyme classifications. Cellulolytic microbes are generally carbohydrate degraders and do not use proteins or lipids as energy sources. Cellulolytic microbe's particularly aerobic bacteria such as Cellulomomas and Cytophagia and the most fungi can use a variety of carbohydrate in addition to cellulose, while the anerobic cellulolytic species have a restricted carbohydrate range, limited to cellulose or its hydrolytic products.

Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials (C. P. Kubicek, 1993) cellulase is a family of at least 3 groups of enzymes (Y. H. Percival Zhang, 2000), endo-(1,4)-*β*-D-glucanase (EC 3.2.1.4) exo-(1,4)-*β*-D-glucanase (EC 3.2.1.91), and *β*-glucosidases (EC 3.2.1.21). The exoglucanase (CBH) acts on the ends of the cellulose chain and releases *β*-cellobiose as the end product; endoglucanase (EG) randomly attacks the internal *O*-glycosidic bonds, resulting in glucan chains of different lengths; and the *β*-glycosidases act specifically on the *β*-cellobiose disaccharides and produce glucose. Although the mechanism of cellulose degradation by aerobic bacteria is similar to that of aerobic fungi, it is clear that anaerobic bacteria operate on a different system (L. M. J. Carvalho, 2003). Cellulosomes located on the cell surface mediate adherence of anaerobic cellulolytic bacteria to the substrate, which thereafter undergo a supramolecular reorganization, so that the cellulosomal subunits redistribute to interact with the different target substrates (E. A. Bayer et al., 2004).

Cellulase producing bacteria were exploited widely for the extraction, purification and characterization of novel cellulase enzymes, because of their higher growth rate, presence of multi enzyme complexes for the synergic action and their wide distribution in different environmental niches. Sacred grove areas of Puducherry, India, hardly have any human influence. Hence this area provides the ample soil sample for the discovery of novel cellulase-producing bacteria. The effective cellulase producing bacteria was isolated, characterized, and identified by 16S rRNA sequencing. In the present study, the optimization of nutritional and environmental parameters for improving the cellulase production was done.

II. MATERIALS AND METHODS

1) Isolation and screening of cellulolytic bacteria

The soil sample was collected at Nallavadu, a sacred grove in Puducherry. The soil was collected at different areas within the sacred grove and pooled together. The samples were collected in sterile container and stored at 4ºC until used. Cellulase producing bacteria were isolated from the sample by serial dilution followed by plating them on a Cellulose Congo Red agar medium. Serial dilution was done by mixing 1gm of soil sample in 100ml of sterile distilled water and further proceeding up to 5 dilutions. 0.1ml of each dilution was plated

on Cellulose Congo red agar (Hendricks and Doyle, 1995) containing the following composition: 2g Carboxymethyl cellulose, 0.25g $MgSO_4.7H_2O$, 0.5g K_2HPO_4, 2g agar, 2g gelatin , 0.2g Congo red in 1000ml at pH 7. The plates were incubated at 37°for 5 days. The appearance of clear hydrolysis zones around the developing bacterial colony indicated cellulose hydrolysis (Wood, 1975). The bacterial colonies were selected depending upon the diameter of the zone. The zone of the bacterial colony having the largest diameter was selected for further study. Purification of selected colonies was done by repeated streaking and stored at 4 °C in CMC slants.

2) Total cellulase assay

Enzyme production

The selected bacterial strains were inoculated in enzyme production medium containing the following composition: 20g Carboxymethyl cellulose, 5g yeast extract 0.2g $MgSO_4.7H_2O$, 5g K_2HPO_4, 10g NaCl in 1000ml at pH7 and incubated overnight at 37 °C in a shaker. After incubation, the culture was centrifuged and the supernatant was used for cellulase assay.

Cellulase assay

The activity of cellulase was assayed using Dinitrosalicylic acid (DNS) reagent, by estimating the reducing sugars released from CMC (Miller *et al*, 1959 with modifications). 0.1ml of crude enzyme was mixed with 0.9ml of acetate buffer [25mM, pH 5.0].1ml of 0.1% CMC was also added and incubated at 50 °C for 10 minutes. After incubation, the reaction was halted by adding 1 ml of DNS reagent. This was followed by the incubation of the tube at 100 °C for 10 minutes in a water bath. The absorbance was read at 550 nm with glucose as standard. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1µmole of glucose/minute.

3) Phenotypic characterization

The cellulase producing bacterial strain was presumptively identified by morphological examination and biochemical characterization. The parameters included Colonial morphology, motility, Gram reactions, Catalase production, Indole production, Citrate utilization, Starch hydrolysis, Urea hydrolysis, H_2S production, and Carbohydrate fermentation.

4) Antibiotic sensitivity test

The susceptibility of strain SG21 to various antibiotics was tested. Disks impregnated with the antibiotics were placed on Muller Hinton Agar plates which had been surface-inoculated with SG21 suspension.

5) Optimization of culture conditions for cellulase Enzyme Production

5.1 Time course induction of cellulase enzyme production.

The bacterial strain was cultured in CMC broth at pH7 and 37°C for 48 hrs. The aliquots of culture were collected at every 6 hr and the growth measurements at 600nm and enzyme assay was performed.

5.2 Effect of pH

Prepare CMC medium of different pH using appropriate buffer like acetate buffer (pH 5), Phosphate buffer (pH 6), Tris buffer (pH 7-8), Glycine NaOH buffer (pH 9-10). The aliquots of culture were collected at every 6 hr and the growth measurements at 600nm and enzyme assay was performed.

5.3 Effect of temperature

The effect of temperature was studied using the same medium (pH 7) at 20, 25, 30, 35and 40 °C. The aliquots of culture were collected at every 6 hr and the growth measurements at 600nm and enzyme assay was performed.

5.4 Effect of carbon and nitrogen source

The effect of different carbon sources such as CMC, xylan, avicel, starch and glucose in 2% concentration was used for study. The effect of different nitrogen sources such as potassium nitrate, urea, yeast extract, ammonium chloride, and peptone was used for study. The aliquots of culture were collected at every 12 hr and the growth measurements at 600nm and enzyme assay was performed.

III. RESULTS AND DISCUSSION

1. Screening of cellulase producing bacteria

A total of 32 cellulolytic bacterial strains were isolated from soil sample collected from sacred groove. Among them, 5 colonies which produced clearest and largest zone with respect to the other colonies on the Cellulose Congo red agar were selected and quantitatively tested for enzyme production. Out of the five bacterial strains, the isolate SG21 had maximum cellulase activity of 2.9U/ml.

2. Phenotypic characteristics of SG21

The cells are rod-shaped, single, Gram-negative and motile, strictly aerobic and the colonies were pale white coloured. The stain showed positive results in catalse test, protease test, urease test, lipase test, starch hydrolysis, simmon citrate test, TSI with glucose utilization, CMC test and indole test. These results correlate that the organisms resembles *Pseudomonas aeruginosa*.

3. Antibiotic sensitivity testing of strain SG21

The strain SG21 was tested against different antibiotics. The organism was sensitive to erythromycin, oxytetracycline, cephalexin, tetracycline, chloramphenicol, amoxillin, ampicillin, vanomycin, gentamycin, methicillin, neomycin, nalidixic acid, ofloxacin and resistant to polymyxin.

4. Optimization of culture conditions for enzyme production.

Effect of Time course induction

The time course induction study of strain SG21 was performed in CMC broth at optimal conditions at every 6h up to 48 hours. The cellulase production was found to be maximum at 18 hrs.

Effect of pH

The strain SG21 was allowed to grow in media of different pH ranging from 5-10. Maximum enzyme activity was observed in the medium from pH6-10.

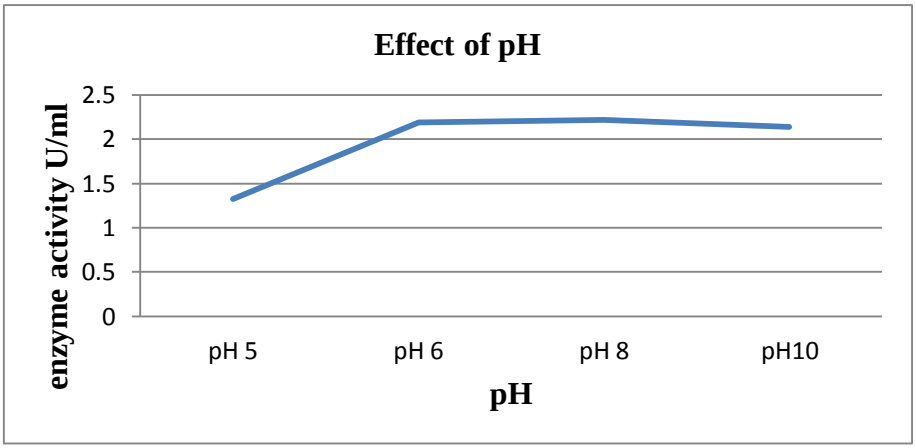

Figure 1- Effect of pH on the growth of *P. aeruginosa* SG21

Effect of temperature

Enzyme activity recorded at different temperatures revealed that the strain SG21 grew well in all the temperature tested and showed maximum enzyme activity at 30 ^{o}C.

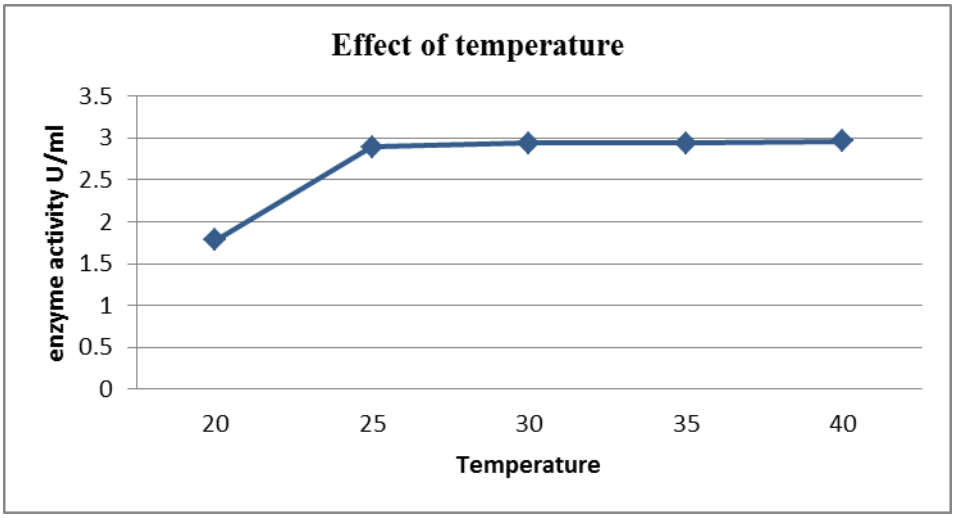

Figure 2 - Effect of temperature on the growth of *P. aeruginosa* SG21

Effect of carbon source

Various carbon sources such as glucose, CMC and Starch were used for the assay. Results revealed that maximum enzyme was produced when CMC was used a substrate.

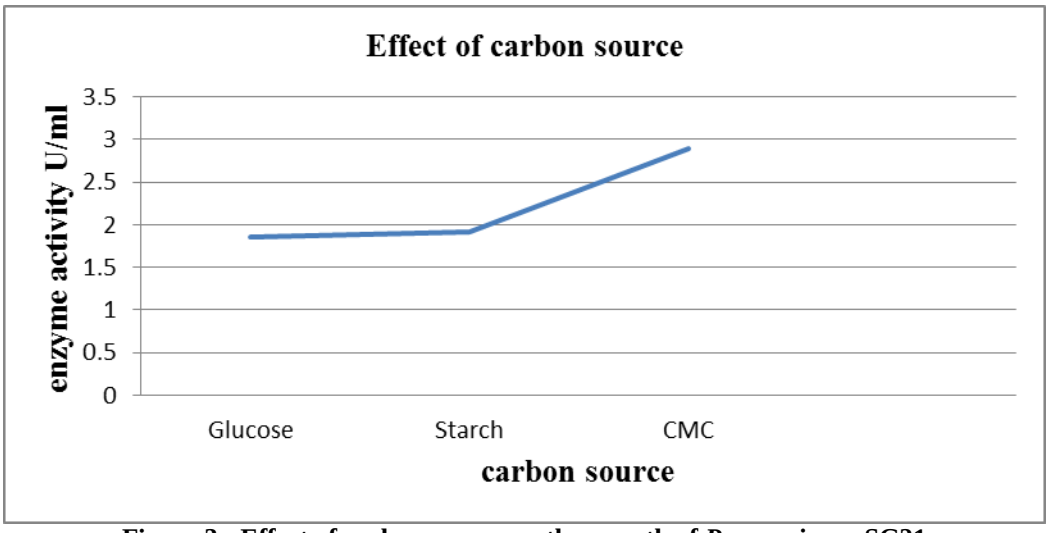

Figure 3 - Effect of carbon source on the growth of *P. aeruginosa* SG21

Effect of nitrogen source

Different nitrogen sources such as ammonium chloride, peptone, urea, potassium nitrate and yeast extract were tested for maximum enzyme activity. Maximum enzyme production was obtained when urea was used a nitrogen source.

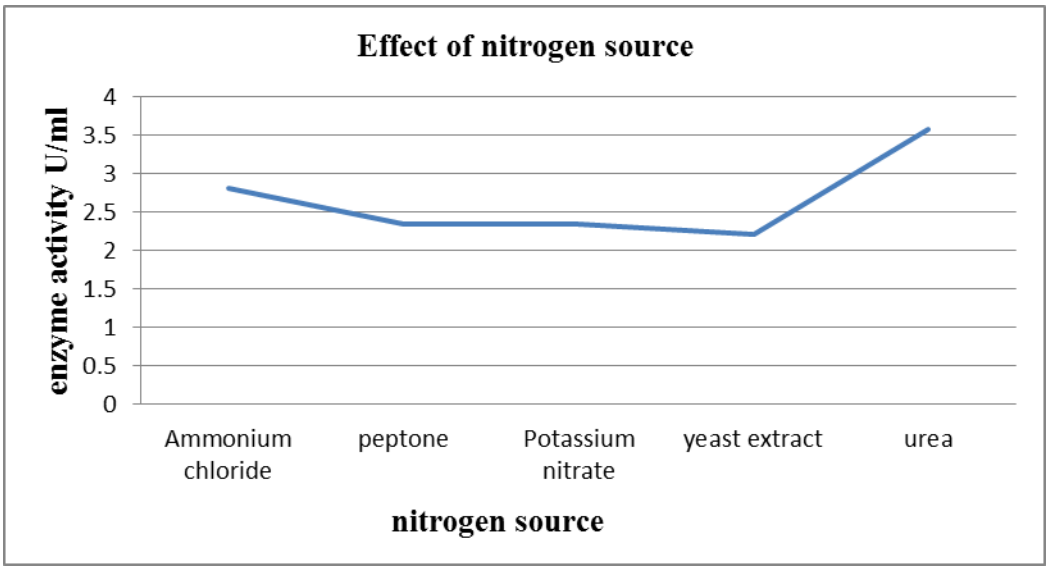

Figure 4 - Effect of nitrogen source on the growth of *P. aeruginosa* SG21

IV. CONCLUSION

Cellulases are being commercially produced by several industries globally and are widely being used in food, animal feed, fermentation, agriculture, pulp and paper, and textile applications. With modern biotechnology tools, especially in the area of microbial genetics, novel enzymes and new enzyme applications will become available for the various industries. Improvements in cellulase activities or imparting of desired features to enzymes by protein engineering are probably other areas where cellulase research has to advance.

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