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RESEARCH ARTICLE

STUDIES ON ISOLATION AND OPTIMIZATION OF CELLULASE PRODUCING BACTERIA FROM ANIMAL DUNG.

Shanthanu M. Raikar and Shakeel Ahmed Adhoni.
P.G. Department of Biotechnology and Microbiology, Karnatak University Dharwad, Karnataka, India.

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Abstract

The present investigation deals with the isolation, screening and characterization of cellulose producing bacteria from termite gut and animal dung, as these are good source of the cellulose digesting environment. The gut bacteria produces extra or intra cellular cellulase for degradation of the cellulose. The microorganisms were isolated and characterised by evaluation of colony morphology and biochemical tests. The organisms were futher screened by Cong red agar media selection for cellulase producing organisms. The organisms (which organism) showing maximum clear zones around the colonies were selected for further studies. Total of thirteen bacterial isolates found to be positive on screening media. Furthermore, a total of eight positive isolates (G2, G3, G4, G5, S1, S3, T1, and T4) were selected for enzyme production and their respective cellulolytic activity was estimated. Enzyme assay for cellulose activity was found to be highest for CMC is S3 (EA=0.112µmol/ml/min) and for cellulose is (EA=0.078µmol/ml/min). The optimization of the isolated bacteria which as the potentiality for the production of cellulose was carried out. Results show that S3 strain was efficient organism for cellulase production with high enzyme activity which can be employed for cellulose degradation in industrial processes.

Introduction:-

Cellulose is the most abundant biomass on the earth for its annual output as high as 1011 tons that is produced by the plants' photosynthesis (Shuler, 1980). The biological degradation and conservation of cellulose constitute a significant component of the natural carbon cycle (Sleat et al., 1984). Cellulose is known as an important reproducible resource for its vast existence and potential applications. However, due to their complicated and insoluble structures, most of them cannot be directly used and are usually discarded, resulting in a serious environmental pollution (Zhengang et.al. 2015). Cellulolytic is a biological process which controlled and processed with cellulase system. Cellulase system consists of three classes of soluble extracellular enzymes, i.e 1, 4-β-endoglucanases, 1,4-β-exoglucanases, and β-glucosidases (β-D- glucoside glucohydrolases or cellobiases) (Shewale 1982). These enzymes hydrolyze cellulose to glucose (Ryu and mandels 1980). One of the best sources for cellulolytic system is symbiotic microorganism in intestinal tract of organism with cellulose as source of metabolizable sugar (glucose). Termites had symbiotic microorganism in their intestinal tract which digests the cellulolytic food (Saxena S et.at., 1993). Cellulase used in various industrial processes, including biofuels such as

Corresponding Author:-Shanthanu M. Raikar.
Address:-P.G. Department of Biotechnology and Microbiology, Karnatak University Dharwad, Karnataka, India.

bioethanol (Vaithanomsat et.al. 2009), plants and agriculture waste processing. To overcome this hindrance, significant efforts are under way to identify novel cellulases. A wide variety of microorganisms have the ability to degrade cellulose, which include aerobic and anaerobic bacteria, white-rot and soft-rot fungi. Fungi are the most studied organisms with respect to the production of cellulolytic enzymes (Humphrey AE 1979). Compared to fungi, bacteria have numerous advantages on an industrial view point like its high growth rate, easy handling and adaptability to various genetic manipulations. Production of extracellular enzymes, especially carboxymethyl cellulase (CMC) by bacteria like Bacillus and Cellulomonas are advantageous for large-scale applications (Noyola and Torre 2001). Most importantly thermophilic, psychrophilic, alkalophilic, acidophilic and halophilic bacteria inhabit a wide variety of environmental and industrial niches that are extremely resistant to environmental stress and they can produce enzymes which are stable under extremely harsh conditions (Sasidharan et.al. 2013). As a result, isolation and characterization of cellulase-producing bacteria will continue to be a principal component of enzyme research.

Materials And Methods:-

Sample collection and extraction

Sample was collected from different sites which includes (i) cellulose feeding organisms, such as termite (Family: Kalotermitidae) residing on woody habitat of botanical garden Karnataka University, Dharwad. (ii) Samples rich in cellulose content like fecal matter of livestock animal's sheep and goat were collected from rural area. Organism *kalotermitidae* was crushed in 0.9% saline solution under sterile condition. Fresh faecal matter of sheep and goat was collected in a clean polythene bag. 1.0 gram of each sample is placed in 9 ml of 0.9% saline, mixed it rapidly and the solution was discarded to remove unwanted flora. The faecal is then again placed in the 9 ml saline solution and crushed, mixed and serial dilution techniques was followed and the dilutions from10^{-1} to 10^{-3} were selected for further studies.

Isolation and Screening of cellulolytic micro-organisms

Samples were collected in a polyethylene bags and brought to the laboratory and stored under sterile conditions. One gram of the sample was crushed and transferred into 9 ml glass tube containing sterile saline. The mixture was mixed by vortex for 2-3 mins for removal of microorganisms. One ml of this sample was plated by serial dilution (up to 10^{-4}) technique amended with CMC agar and incubated at 37°c for 24 -48 hours. Bacterial cultures grown on CMC slants were cultured on basal mineral salt medium (BSM).

Detection of cellulase activity was performed on the culture plate using Cong red solution (0.1mg/ml) was added to the each plate and the colonies surrounded by transparent zone were considered as positive for cellulase producing bacteria, the diameter of zone was also measured, the colonies showing maximum diameter was subsequently purified by repeated streaking on to new plate containing CMC agar medium for three times and used for further studies.

Biochemical tests

On the basis of the shape and Gram's nature of the organism, biochemical tests were performed.

Indole test

These tests were performed by inoculating a loop-full of culture in tryptophan broth and then incubated at 37^0 C. After incubation for 24 hours kovacs reagent was added to the tubes. If the cherry red colour ring is formed indicates positive for indole production.

MR test

These tests were performed by inoculating a loop full of culture in MRVP media and then incubated at 37^0 C for 3-5 days. After incubation period half of the culture was transferred to a clean tube and other tube of culture was used to conduct VP test. After addition of methyl red to one tube, if red coloration occurs it indicates positive result for MR test to other, after addition of α-naphthol and KOH pink coloration indicates positive test (Experiments in microbiology, plant pathology and biotechnology by K R Aneja 2003).

VP Test
These tests were performed by inoculating a loop full of culture to VP media and then incubated at 37°C for 24 hours. After incubation addition of α-naphthol and KOH, pink coloration indicates positive test (Johnson and Case, 2007).

Citrate
Two sterile test tubes with Simmon's citrate agar media was prepared, one inoculated with the culture and one tube is kept as control incubated at 37 °C for 24 hours, if the color of media changes from green to blue it indicates positive test(Experiments in microbiology, plant pathology and biotechnology by K R Aneja 2003).

Catalase test
To 3 ml of hydrogen peroxide, add a loop full of culture using inoculating loop on a clean glass slide, evolution of effervescence indicates positive test (Experiments in microbiology, plant pathology and biotechnology by K R Aneja 2003).

Acid and gas production
In lactose fermentation broth, place durham's tube in inverted position without any air bubble, inoculate a loop full of culture into it and incubate at 37 °C for 24 hrs. If media turns yellow, it indicates acid production and if bubbles are formed in Durham's tube it indicates gas production (Experiments in microbiology, plant pathology and biotechnology by K R Aneja 2003).

Starch hydrolysis
Starch Agar medium was prepared and inoculated with microorganism and incubated for 24 hrs at 37 °C. After incubation, the plates were treated with iodine to conform the hydrolysis of starch.

Mannitol test
This test determines ability of organism to utilize mannitol as source of carbon. A slant of mannitol agar in clean and sterile test tube is streaked with a loop full of culture and incubated for 24hrs. If the color changes from pink to yellow, it refers positive to mannitol test.

Urease test
These tests were performed by inoculating a loop full of culture in urease agar media and then incubated at 37^0 C for 24 hours. If the media turns into pink colour shows positive test for urease.

Optimisation of Cellulose
Effect of ph
To determine the optimum pH for cellulase production, Media containing 0.5% CMC with different pH, i.e., 4, 7and 9 was inoculated and incubated at 37°C. Whole flask samples were withdrawn and cellulase activity in the supernatant was assayed.

Effect of temperature
To determine the optimum temperature for cellulase production media containing 0.5% CMC was prepared with optimum pH fixed earlier for each culture was incubated at 37°C and Room temperature. Whole-flask samples were used for the cellulase assay.

Enzyme assay (CMC and Cellulose substrate) by DNS method:
Culture broth was centrifuged at 5000rmp for 5min at 4°C and Cellulase activity in the supernatant was measured. The reaction mixture contains 1ml of 0.5% substrate (CMC and Cellulose), 0.1M .Acetate Buffer pH 5.0 and 1 ml of crude enzyme was added to and incubated for 30 min at 37°C, than add 1ml of DNS Reagent (3, 5-dinitro salicylic acid) kept on boiling water bath for 5min and cooled. The absorbance was measured at 540nm. (UV visible spectrophotometer, Model: Hitachi-U2900). One unit of cellulase activity is defined as the quantity of cellulase required to liberate 1.0 μmol of glucose equivalents per min under the assay condition.

Results And Discussion:-

For isolation of cellulose degrading bacteria, termites were collected from woody habitat, as it contains cellulose degrading bacteria in its gut and digestive tract. Faecal matter of Sheep and Goat was so collected, because of their

grassy diet and multiple stomach which also inhabits cellulitis bacteria in their digestive tract. The colony morphology was studied in detail and the results are presented in (Table.1), followed by biochemical tests performed for the isolated microorganisms (Table. 2) .The collected samples were then subjected for serial dilution technique and were inoculated on to a plates containing CMC agar medium and incubated at 37°c for 24-48 hours (Figure. 1). After incubation Cong red test was performed for screening of the cellulolytic organisms. The organisms (which organism) showing maximum clear zones around the colonies were selected for further studies. Total of thirteen bacterial isolates found to be positive on screening media (cellulose Cong red agar) by producing clear zone around their colonies (Figure. 2). Furthermore, a total of eight positive isolates (G2, G3, G4, G5, S1, S3, T1, and T4) were selected for enzyme production and their respective cellulolytic activity was estimated. Enzyme assay for cellulose activity was found to be highest for CMC is S3 (EA=0.112µmol/ml/min) and for cellulose is (EA=0.078µmol/ml/min) (Graph.1). The optimization of the isolated bacteria which as the potentiality for the production of cellulose was carried out and results are shown in Table.3 and Table.4.

In recent years, more attention has been directed towards screening of novel microbial strain that has broad spectrum enzyme activity. The high cost of cellulase production and low enzyme activities limit their industrial use such as bio-fuel production, bio fertilizers, and textile industry. So effort are to be taken to economize and increase the yield of cellulase production by media optimization (Ma .J et al., 2015) and hence isolation, characterization and media optimization for cellulase producing bacteria remain to be an important area of bio fuel research (P. Gupta et al., 2012)

In the present study, we focused on isolation of cellulase producing bacteria with an industrial and Agricultural perspective. So we collected faecal matter of livestock animals such as Sheep and Goat from rural area Haliyal and Malamaddi, Dharwad as our site for sample collection. We selected this source for our study because these animals feed on wide variety of plant biomass which is rich in cellulose diversity able to degrade different types of cellulose as they contain most of cellulase producing microorganisms able to degrade cellulose and we collected termite belonging to family *kalotermitidae* in woody habitat from Botanical Garden Karnataka University Dharwad. We selected these samples because termite has symbiotic microorganisms in Gut and digestive tract which helps in digesting the cellulose containing food. Collected samples were serially diluted and plated on minimal salt media (CMC agar media), (Shankar et al., 2011) pure cultures obtained were characterized morphologically ,screened on Cong red agar media for selection of cellulase producing organism. Out of 9 isolates 3 isolates (isolate1 S3, isolate2 G3, isolate3 T4) showed maximum zone, further 3 cultures used for biochemical characterization. Pure culture obtained were inoculated in two broth containing two different substrates 1% CMC & 1% cellulose for cellulase enzyme assay, all 9 isolates were checked for enzyme activity for confirmation. Same 3 isolates (isolate S3, isolate G3, isolate T4) showed maximum enzyme activity, based on standard calibration curve by DNS method. We optimized isolated organisms by varying parameters such as pH (4, 5, 7, 9, 10) and Temperature (4, 18, 30, 37 ^{0}C) by using different substrates CMC Cellulose at different time intervals 30 & 60 minutes. Earlier studies have shown maximum enzyme activity at pH 7 and at temperature from 20-50^0 C for thermostable alkaline cellulase producing bacterium strain from garbage dump (Zhen gang Ma et al. 2015). Further crude samples subjected for SDS-PAGE .Overall isolate S3 showed maximum enzyme activity. Hence we can conclude that the isolate can be used for production and isolation of cellulose which can be used for industrial processes.

Table 1:-Colony morphology of isolated organisms.

Sample	Size (mm)	Shape	Margin	Colour	Elevation	Optical property	Gram staining	Organism
G1	4	Round	Regular	Watery	Raised	Shiny	Gm +ve	Cocci
G2	3	Round	Regular	Creamy	Raised	Shiny	Gm –ve	Bacilli
G3	2.5	Round	Regular	Yellow	Raised	Dull	Gm +ve	Cocci
G4	3	Round	Regular	Yellow	Convex	Shiny	Gm +ve	Cocci
G5	3	Round	Irregular	Brownish	Flat	Shiny	Gm –ve	Bacilli
S1	3.5	Irregular	Irregular	Pink	Flat	Dull	Gm –ve	Bacilli
S2	4	Round	Circular	White	Convex	Dense	Gm +ve	Cocci
S3	2	Round pinpoint	Circular	Light orange	Convex	Dense	Gm +ve	Cocci
S4	3	Irregular	Irregular	Light brown	Flat	Shiny	Gm +ve	Bacilli

T1	2	Round	Regular	Creamy	Raised	Shiny	Gm +ve	Cocci
T2	3.5	Irregular	Irregular	Yellow	Flat	Opaque	Gm –ve	Cocci
T4	3	Round	Regular	Buff colour	Flat	Opaque	Gm +ve	Cocci

Table 2:-Biochemical test of the selected organisms

Organism	**S3**	**G3**	**T4**
Gram's staining	Gm +ve cocci	Gm +ve cocci	Gm +ve short rods
Endospore staining	Non sporulating	Non sporulating	Non sporulating
Indole test	+ve	+ve	+ve
Methyl red test	+ve	-ve	+ve
VP test	+ve	+ve	-ve
Citrate test	-ve	-ve	-ve
Catalase test	+ve	+ve	+ve
Acid and gas	+ve	+ve	-ve
Starch hydrolysis	+ve	+ve	+ve
Mannitol test	+ve	+ve	+ve
Urease test	+ve	+ve	-ve

Table 3:-Enzyme activity on CMC as a substrate.

Sample	**CMC (ml)**	**Buffer (ml)**	**Enzyme (ml)**	**Incubation cmc (min.)**	**DNS**	**O.D**
G2	1.0	1.0	1.0	30	1.0	0.139
G3	1.0	1.0	1.0	30	1.0	0.336
G4	1.0	1.0	1.0	30	1.0	0.20
G5	1.0	1.0	1.0	30	1.0	0.186
S1	1.0	1.0	1.0	30	1.0	0.281
S3	1.0	1.0	1.0	30	1.0	0.455
T1	1.0	1.0	1.0	30	1.0	0.245
T4	1.0	1.0	1.0	30	1.0	0.284

Table 4:-Enzyme activity on Cellulose as a substrate.

Sample	**Cellulose (ml)**	**Buffer (ml)**	**Enzyme (ml)**	**Incubation cellulose (mins)**	**DNS (ml)**	**O.D**
G2	1.0	1.0	1.0	60	1.0	0.137
G3	1.0	1.0	1.0	60	1.0	0.194
G4	1.0	1.0	1.0	60	1.0	0.181
G5	1.0	1.0	1.0	60	1.0	0.139
S1	1.0	1.0	1.0	60	1.0	0.100
S3	1.0	1.0	1.0	60	1.0	0.212
T1	1.0	1.0	1.0	60	1.0	0.154
T4	1.0	1.0	1.0	60	1.0	0.206

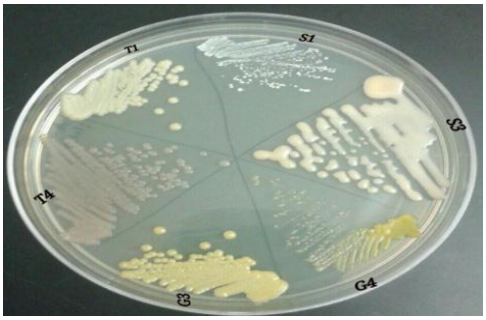


Figure 1:-Pure culture plates of the isolated microorganisms.

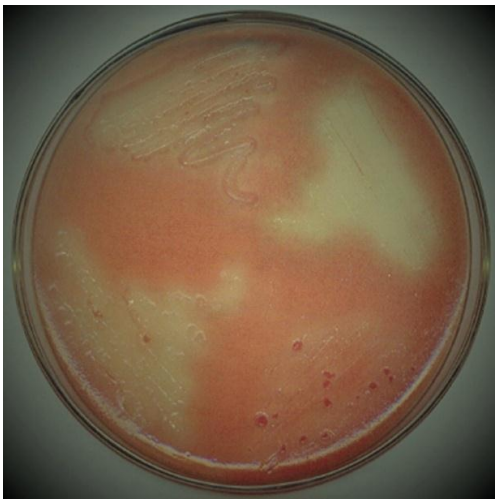
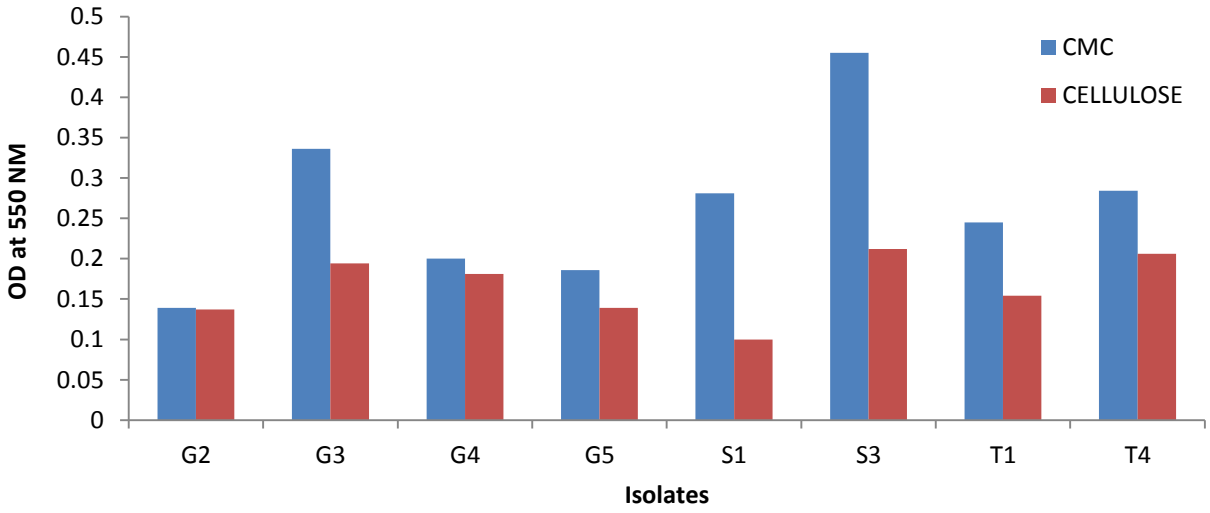


Figure 2:-Bacterial isolates found positive on Cong red agar.

Graph 1:-Comparative analysis of cellulase activity of isolates on different substrates



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