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ISOLATION AND CHARACTERIZATION OF CELLULASE PRODUCING PARACOCCUS PANTOTROPHUS FMR19 (JX012237) FROM GOAT RUMEN FLUID AND ITS EFFECTS ON pH, TEMPERATURE AND CARBON SOURCES

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ABSTRACT

The cellulase producing bacteria was isolated from goat rumen fluid and characterized by morphological and biochemical analysis. The organism was identified as *Paracoccus pantotrophus* by 16S rRNA sequencing analysis and it was submitted to GenBank and the Accession Number was given as JX012237. The *Paracoccus pantotrophus* was grown on Carboxyl Methyl Cellulose (CMC) agar at various optimum conditions such as pH, temperature and carbon sources. Maximum cellulase production was obtained at pH 4 with 3600 Units/mg and the temperature at 40°C with 1200 Units/mg. Cellulase synthesis increased in the presence of Xylose and Maltose, liberated 3636 Units/ mg and 3040 Units/mg respectively. In Filter Paperase activity the Xylose produced maximum cellulase activity with 3700 Units/mg. The purpose of the current investigation was to screen facultative anaerobes isolated from rumen fluid in order to study its suitability with regard to waste treatment from the tanneries.

Keywords: Rumen Fluid, *Paracoccus pantotrophus*, Cellulase, Carboxyl Methyl Cellulose, Maltose, Xylose.

I INTRODUCTION

Cellulose is the major form of stocking of glucose which is obtained through photosynthesis and also the major component of solar energy conversion to the biomass. Cellulase is a linear polymer and it can be degraded into glucose by cellulase. Cellulose is a highly ordered structure, it is very hard to be degraded and it is unusable and stocked in nature as waste. The capacity of cellulase in degrade the natural cellulose implies the synthesis of entire cellulolytic system. Cellulose, enormous potential as a renewable source of energy was recognized only after cellulose degrading enzymes ‘cellulases’ had been identified [5].

Cellulases have enormous potential in industrial applications. Glucose produced from cellulosic substrate could be further used as substrate for subsequent fermentation or other processes which could yield valuable end products such as ethanol, butanol, methane, amino acid, single cell protein, etc., [24]. *Bacillus sp.* was isolated from cow dung which showed good cellulase production. Carboxyl Methyl Cellulose was found to be the best substrate for cellulase production compared to coir waste and saw dust as substrates [23]. From the paper and timber sawmill industrial wastes, the Aspergillus niger produced cellulase enzyme [7].

The rumen is a special digestive vessel, within which the digestion of cellulose and other plant polysaccharides occurs, through microbial activity. Microbes produced in the reticulo-rumen are also digested in the small intestine. Fermentation continues in the large intestine in the same way as in the reticulo-rumen [22]. Superior biological efficiency has been claimed for the goat [11] and its superior digestive efficient has been mostly associated with utilization of plant structural polymer [12,9] and with a larger digestive capacity compared to sheep [15].

All most all the glucose produced by the breaking down of cellulose and hemicellulose is used by microbes in the rumen, and as such ruminants usually absorb little glucose from the small intestine [13]. The bacteria *Fibrobacter succnigenes, Bactroide succinogenes, Clostridium ochheadii, Bacillus licheniformis* and *Streptococcus* anaerobes are generally regarded as the predominate cellulolytic microbes in the rumen [10].

In recent years many rumen microbes have been isolated and characterized by sequence analysis of 16S rRNA. Prokaryote diversity in the rumen of yak (Bos grunniens) and Jinna cattle (Bos Tauras) were estimated by 16S rDNA homology analysis [8]. Some of the microbes have also been recommended as feed additive for improving the overall growth or production of animals [19].

The enzymes produced by rumen bacteria may contribute to the breakdown of switch grass, a renewable biofuel energy source [2]. The *Pseudomonas aeruginosa, Bacillus, Penicillium, Aspergillus, Mucor and Fusarium species* were isolated from the rumen of cow, sheep and goat. These organisms are active in cellulose breakdown [20]. The *Butrivibrio fibrisolvens, Streptococcus sp.,* and *Clostidium aminophilum* were isolated from cattle rumen fluid and they have better cellulase enzyme activity [17].

The present study was aimed to screen the goat rumen bacterial isolate for the cellulolytic ability. Furthermore, this study aims to provide better understanding of condition for the production and activity of cellulase by the bacterial culture from goat rumen fluid.

II MATERIALS AND METHODS

2.1 Sample Collection

Rumen fluid was freshly collected from slaughter house near Chennai and filtered through a layer of muslin cloth.

2.2 isolation and Screening of Cellulase producing bacteria

1ml of filtrate was used for serial dilution, 100 µl of each dilution (10^{-6} to 10^{-9}) was cultured by spread plate using Hungate's medium [14]. Three isolates were obtained and designated as M_1, M_2 and M_3.

2.3 Enrichment of pure culture

The colonies were sub cultured in minimal agar supplemented with 1% Carboxyl Methyl Cellulose (CMC) agar containing $NaNo_3$ 2.0, KH_2Po_4 1.0, $MgSo_4$.$7H_2O$ 0.5, KCl 0.5, Carboxymethyl cellulose sodium salt 10.0, peptone 0.2, agar 17.0 plates were incubated at 37˚C for 5 days to allow the secretion of cellulose. After incubation the colonies were screened for cellulase production by Congo red Assay [3]. The formation of a clear zone of hydrolysis indicated cellulose degradation. The M_2 isolate produced comparatively maximum zone of clearance than the other isolates.

2.4 Identification for Cellulase producing bacteria

The M_2 isolate was presumptively identified by means of morphological examination, biochemical characteristics and sugar fermentation test [6].

2.5 16s rRNA Sequencing

The genomic DNA was sequenced from M_2 isolate and amplified by using primer 16S-F=8f (5`GAGTTTGATCATGGCTCAG-3`)

16S-R=1495r

(5`CTACGGCTACCTTGTTACG-3`) [1] and the sequence was submitted to GenBank for Accession number.

2.6 Enzyme Activity Assay

2.6.1 Preparation of Crude Enzyme

After incubation, the culture was centrifuged and supernatant was used as a source of crude enzyme [16]. The crude enzyme solution was utilized for determination of enzyme activity.

2.6.2 DNS Method

Cellulase activity was measured by **a** 3, 5-dinitrosalicylic acid **(**DNS) method, through determination of reducing sugars liberated [18]. 500 µl of CMC solution, 500 µl of crude enzyme and 500 µl of 0.05M Citrate buffer pH 4.8 were incubated for 30 min at 50˚C before adding 2 ml of DNS solution. The treated samples were boiled for 15 min prior to cool down in cold water for color stabilization. The optical density was read at 540 nm against reagent blank by a UV-Spectrophotometer. 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 1 µmol of glucose per minute.

2.6.3 Filter Paperase (FPase Activity)

The activity of FPase was assayed according to the method explained by Wood and Bhat, 1998 with some modifications. The methods are similar to the CMCase assay method, but the substrate used was Whatman no.1 filter paper strip (1*3 cm) soaked in 1.8 ml 0.05M Sodium Citrate buffer (pH 4.8). The samples were incubated at 40°C for 60 min. The reaction was terminated by adding 3 ml of dinitrosalicylic acid (DNSA) reagent and by subsequently placing the reagent tubes in water bath at 100°C for 15 min. One ml of Rochelle salt solution (40 g Rochelle salt in distilled water to make the volume 100 ml) was then added. The absorbance was recorded at 575 nm against the blank (of 0.05M Sodium Citrate Buffer). One unit of FPase activity was determined as 1µmole of glucose liberated per ml enzyme per minute.

2.7 Effect of pH, Temperature and Carbon Sources on Cellulase Production

The cell free supernatant was used. The enzyme activity was determined using DNS method at varying pH values from 3 to 8 and temperature varying from 30 to 80°C. Carbon source such as Glucose, Sucrose, Lactose, Maltose and Xylose (1g/l, w/v) were added separately as a sole carbon source. All factors influence on enzyme activity was determined by measuring cellulase activity.

III RESULT AND DISCUSSION

3.1 Screening for cellulase producing bacteria

It was observed that the isolate M_2 showed comparatively maximum zone of cellulase agar clearance than the other isolates by Congo Red Assay.

3.2 Identification for cellulase producing bacteria

Morphological Characteristics of *Paracoccus*

Colony Morphology

Configuration	: Slimy
Margin	: Irregular
Elevation	: Slightly Raised
Pigment	: Cream
Opacity	: Opaque
Gram's Reaction	: Positive
Cell Shape	: Cocci
Arrangement	: Single & Pairs

Spore & Motility

Endospore	: Non-Spore former
Motility	: Non-motile

The isolate M_2 appeared slimy, pale color colonies on Hungate's media. A microscopic examination of the isolate revealed that it was Gram Positive cocci shaped, non-spore forming and non-motile.

The bacteria appeared single or in pairs (Fig.1).

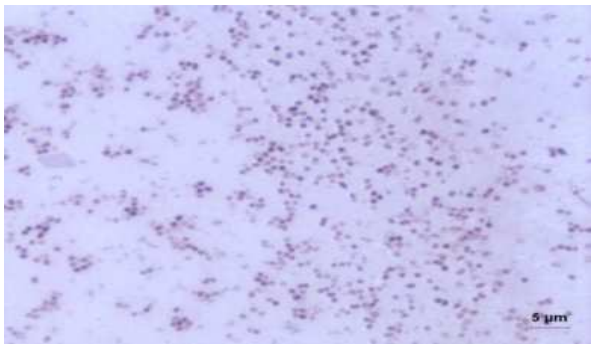

Fig. 1. Photomicrograph of Paracoccus pantotrophus FMR 19 (1000x)

3.3 Biochemical Characterization and Sugar Fermentation Test

The isolate M_2 was found to be positive for Citrate, Catalase and Oxidase and Negative for indole, methyl red, Voges Proskeur and hydrogen sulphide (H_2S) production. Acid Butt and no gas production in triple sugar iron test. The isolate M_2 fermented glucose, fructose, maltose, sucrose and starch and did not ferment lactose, xylose and arabinose. The results of the Biochemical characterization & Sugar fermentation was compared with Bergey's Manual [4] and the isolate was identified as the Genus ***Paracoccus***.

Biochemical Characteristics

Test	**Result**
Indole	-
Methyl Red	-
Voges Proskauer	-
Citrate	+
Catalase	+
Urea Hydrolysis	+
Oxidase	+
H_2S production	-
Triple Sugar Iron Test	Alkaline Slant Acid Butt, No Gas

Sugar Fermentation Test

Glucose	+
Fructose	+
Lactose	-
Maltose	+
Sucrose	+
Starch	-
Xylose	-
Arabinose	-

3.4 16S rRNA sequence analysis

The PCR product of 16S rRNA was sequenced and the sequence result was submitted to BLAST-N to obtain the possible hits based on 97-99% similarity. The isolate belongs to the genus ***Paracoccus***. The 16S rRNA sequence of the isolate M2 (***Paracoccus pantotrophus* FMR 19**) was submitted in GenBank and obtained the Accession number (**JX012237**).

3.5 Phylogenetic Tree

The phylogenetic tree analysis was performed using CLUSTAL-W program to validate the results obtained from BLAST-N. The results are correlated with the BLAST-N results confirming that 16S rRNA have a close relationship with ***Paracoccus pantotrophus***. Fig.2 shows the phylogenetic tree obtained by neighbor-joining method with 97-99% sequence similarity.

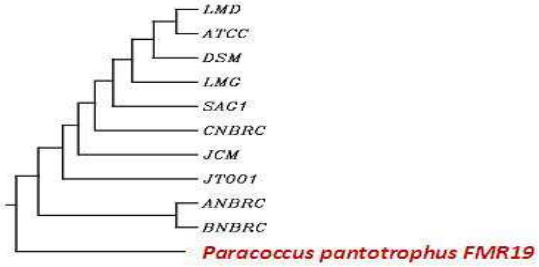

Fig. 2.Comparitive sequence analysis of 16S rRNA from ***Paracoccus pantotrophus FMR19*** and representative strains of the genus ***Paracoccus*** from GenBank, using the neighbor-joining method.

3.6 Enzyme Activity

Table:1 Effect of pH on cellulase production

pH Range	**Cellulase Production**
3	+
4	+++
5	++
6	+
7	-
8	-

The isolate M_2 (Table1) was found to have maximum cellulase activity at pH 4 with 3600 Units/mg in the third day. The least cellulase enzyme activity was found to be at pH 6 with 125 Units/mg (Fig.3).

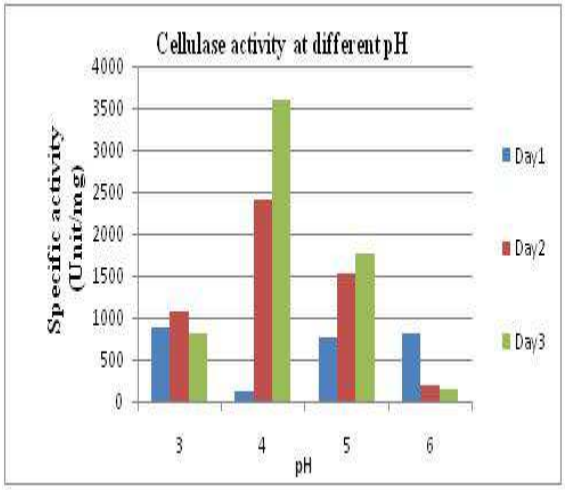

Fig.3. Effect of pH on cellulase production

Table:2 Effect of Temperature on cellulase production

Temperature	Cellulase Production
30°C	+
40°C	+++
50°C	++
60°C	-
70°C	-
80°C	-

The isolate M_2 (Table 2) was found to have maximum cellulase activity at 40°C with 1200 Units/mg. The least cellulase enzyme activity at 30°C with 521 Units/mg (Fig.4).

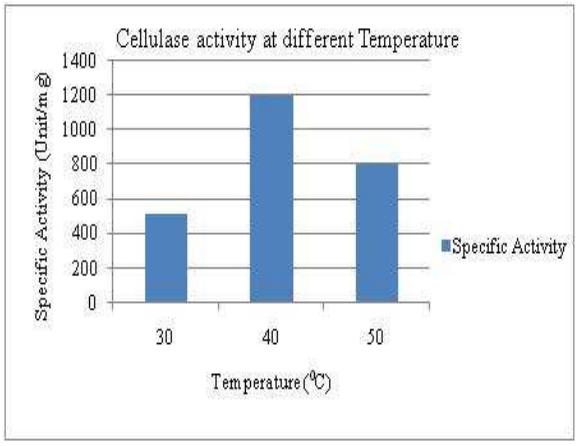

Fig.4. Effect of temperature on cellulase production

Table:3. Effect of Carbon Source on cellulase production

Carbon Source	Cellulase Production
Glucose	++
Sucrose	+
Maltose	++++
Xylose	++++
Lactose	+

The isolate M_2 (Table 3) was found to be maximum cellulase activity in Xylose and Maltose on the day first. Xylose liberated 3636 Units/ mg of protein and Maltose liberated 3040 Units/mg of protein, used as sole carbon source(Fig.5).

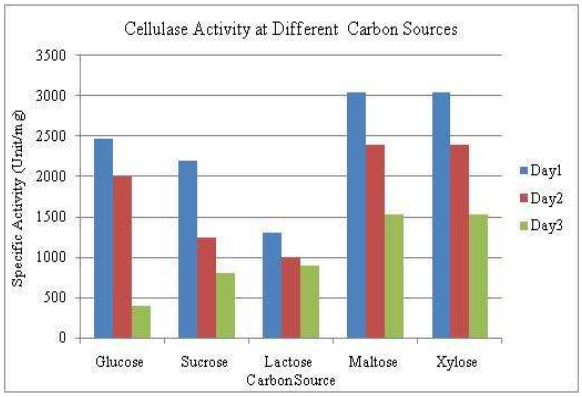

Fig.5. Effect of Carbon Source on Ccellulase production

3.6.4 FILTER PAPERASE ACTIVITY

Out of 5 different carbon sources, the Xylose produced maximum cellulase activity on the first day they liberated 3700 Units/mg protein, whereas the maltose produced high cellulase activity 3100 Units/mg protein. But the lactose produced the least cellulase activity (fig.6). Rumen cellulolytic microbes extracted showed 40.02% yield and a specific activity of 718.4 U/mg. It was active at pH 6 and 40°C while stable from pH 5 to 7 and 20 to 40°C [21] .

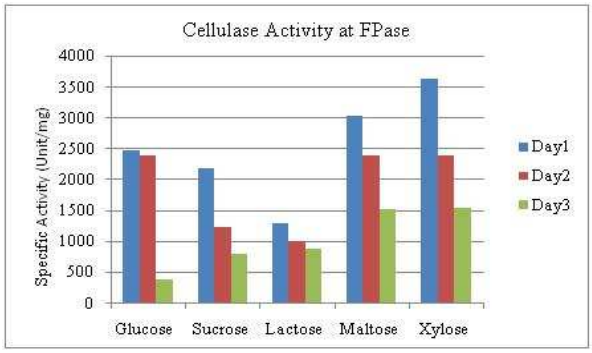

Fig.6. Cellulase activity at Fpase

CONCLUSION

The present work was carried out to optimize the nutritional and environmental parameters for improving cellulase production by cellulolytic bacteria. The cellulase producing *Paracoccus pantotrophus* FMR19 was isolated from goat rumen fluid and characterized by biochemical, sugar fermentation and 16S rRNA sequence analysis. The high titre activity of *paracoccus pantotrophus* produced cellulase at pH 4, temperature at 40°C with xylose and maltose as a sole carbon source. It is the first report of acid tolerant cellulase producing *Paracoccus pantotrophus* from goat rumen fluid.

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