International Journal of Research Instinct
(Peer-reviewed Journal)

ISSN ONLINE 2348-2095
Research Article

Isolation of cellulolytic bacteria and production of cellulase from coir pith

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ABSTRACT

Isolation and screening of cellulolytic bacteria was done in CMC supplemented agar plates, on inoculating fifty bacterial isolates only twelve strains showed cellulolytic activity. Bacterial strain no SACT9 showed higher cellulase activity of 0.480 (U/ml). Isolated cellulolytic bacterial strain no SACT9 showed following characteristics such as Gram negative, rod shape, motile in nature and showed positive results for indole utilization, voges proskauer, citrate utilization, glucose fermentation, catalase, oxidase and nitrate reduction tests which was identified as *Pseudomonas sp*. Coir pith rich in carbon source was selected as substrate for cellulase production. Coir pith was pre-treated with 3 % HCl and bleached for the breakdown of lignin into cellulose and it contains 97 mg of cellulose .On optimization of media for cellulase production, optimum pH was found be 6 and temperature for higher cellulase production found at 40 ° C and 72 hrs incubation. The cellulase enzyme was produced using coir pith under optimum fermentation conditions and the amount of cellulase activity was found to be 0.960 (U/ml).

Keywords: Cellulose, Cellulase, Bacteria, Coir pith

1.Introduction

Cellulose is the major constituent of plant matter and thus represents the most abundant organic polymer on Earth. Cellulose makes a large fraction of the plant dry weight, being typically in the range of 35-50 % **(Sanchez and Cardona, 2008)**. In recent years, accumulation of waste cellulose is increasingly realized as an environmental problem and the utilization of the waste cellulose has become a welcome issue. It is the most widely used natural substance and has become one of the most important commercial raw materials **(Lynd *et al.,* 2002).**The cellulose polymer is composed of crystalline and amorphous regions. Cellulose consists of D-glucose units, which condense through β (1, 4)-glycosidic bonds **(Klemm *et al.,* 2005)**.

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials. Complete enzymatic hydrolysis of cellulose requires the synergistic action of three types of enzymes, namely Cellobiohydrolase, endoglucanase or carboxymethylcellulase (CMCase) and β-glucosidases or cellobiase **(Choi *et al.*, 1978)**. Cellulolytic microorganisms are found among extremely variegated taxonomic groups. Cellulolytic microorganisms can be found in all biota where cellulosic waste accumulates **(Smith and Bryant, 1979)**. The cellulase is released into the substrate and the free enzymes start hydrolyzing the cellulose. The glucose to a length of maximum four glucose molecules are taken up by the microorganism and either used directly or cleaved further via intracellular hydrolases. Most of the facultative anaerobic bacteria that produce non-complexed cellulase systems are most often used in the industrial production of cellulolytic enzymes, because the secreted enzymes can easily be harvested **(Lynd and Weimer, 2002)**.

Cellulases have attracted considerable attention in recent years due to their great biotechnological and industrial applications. Conversion of food, industrial and agricultural wastes in to valuable sugars is the potential use of cellulase enzymes (**Bothast and Saha, 1997)**. Cellulases are being studied because of their application in the hydrolysis of cellulose, the most abundant biopolymer and potential source of utilizable sugars. These serve as raw materials in the microbial production of a wide variety of chemicals, food and fuel. Cellulose is hydrolysed by a multi component cellulase system of microorganisms to glucose. **(Penttila *et al.*, 1986)**.

Coir pith is an organic matter which originates from the tropical hemisphere, especially from south-east Asia where coconut oil production is extensive. For professional oil winning companies, the husk of the nut is a waste product. These coconut husks mainly consist of coir pith and coir fibers. Initially, coir pith was considered a waste but now research has established widespread applications for it. Being an agro-waste from the coir industry, coir pith serves as a renewable source. Accumulation of coir pith near coir retting factories causes solid waste pollution problems mainly due to the ligno-cellulosic compounds present in them. The lignin (31 %) and cellulose (27 %) that they contain is responsible for their slow degradation. Over the last few years, environmental concerns have increased attention toward using coir pith as an alternative substrate with orientation towards agricultural needs. Thus, the conversion of such agricultural wastes into useful products may decrease the problems they cause. This study deals with the utilization of Coir pith for cellulase production. But the lignin present in the coir pith was observed to interfere with hydrolysis by irreversibly binding the hydrolytic enzymes, thereby blocking access to cellulose. Pre-treatment of coir pith increases the crystallinity of

cellulose, thus removing lignin and enabling its enzymatic degradation. In addition, pre-treatment may increase the surface area of the cellulose thereby enhancing its reactivity with the enzyme and thus its transformation **(Anuradha *et al.*, 2014)**.

2. Methodology:

2.1 Sample collection:

Coir pith was collected from agriculture fields in Samayapuram village,Trichy. The dried sample was crushed and powdered in ball mill.

2.2 Cellulose extraction with acid pre-treatment:

Acid pretreatment of coir pith was carried out by using dilute hydrochloric acid. Ten grams each of cellulosic wastes was soaked in 100 ml of 3 % HCl separately and incubated at room temperature for 10 hr with an agitation of 150 rpm and autoclaving at 121 ^{o}C for 30 min. The residues were collected and washed extensively with tap water until neutral pH was reached, filtered and dried at 65 ^{0}C for two days **(Ibrahim *et al.*, 2010)**

2.3 Estimation of cellulose content in substrate:

Cellulose content was estimated by the method of **Uppdegraff (1969)**. Hundred milligram of pre-treated of cellulosic wastes were added with 5 ml of Nitric reagent and boiled and cooled. It was centrifuged at 5000 rpm for 5 min. The pellet was washed with distilled water. Ten ml of 67 % sulphuric acid was added. One ml of the sample was diluted to 100 ml. To 1 ml of the each diluted solution, 10 ml of freshly prepared ice cold Anthrone reagent was added and boiled in a boiling water bath for 10 min at 100 ˚ C. Absorbance was recorded at 600 nm.

2.4 Isolation of bacterial strain:

Bacterial strains were isolated from soil sample collected from Srimad Andavan Arts and Science college (Autonomous) garden, Tiruvanaikovil,Trichy. Soil samples were collected and serially diluted up to 10^{-5} to 10^{-7} and spread on agar plates followed by incubation at 37 ° C for 24 to 48 hrs.

2.5 Screening and identification of cellulolytic bacteria:

Screening and selection of cellulolytic bacteria was done as described by Wood (1980). 0.6 % CMC supplemented with Luria agar medium was prepared. Wells of 5 mm diameter were cut on the agar. The newly isolated bacterial colonies were individually cultured in the Luria broth and the cells were harvested and loaded onto each well cut on the agar medium. The plates were incubated for 24-48 hrs. After incubation, the plates were flooded with 0.2 % (w/v) Congo red solution for 15 min, and then destained by washing the plate with 1 M sodium chloride solution for several times. Cellulolytic bacterium was identified through biochemical tests. The utilization

of sugars under aerobic and anaerobic conditions was tested by the mellow test through bacterial minimum biochemical reaction tubes (Himedia, Mumbai) **(Dong and Cai, 2001)**.The tests were conducted for sucrose, lactose, glucose, maltose, D-mannitol raffinose, arabinose, D-xylose inulin, oxidase, Catalase, indole production, methyl red, hydrogen sulphide production, Voges-proskauer, citrate, lysine decarboxylase, gelatin hydrolysis, Esculin hydrolysis, acetate utilization, Nitrate reduction and ortho-Nitrophenyl-β-galactoside (ONPG) **(Sneath,1986)**.

2.6 Media components optimization

2.6.1 Effect of pH

The optimum pH was determined by preparing the production medium in various pH such as 2, 4, 6, 8 and 10 and culture was inoculated and incubated.

2.6.2 Effect of Temperature

The optimum temperature was obtained by incubating the production media at various temperatures such as 20 ° C to 80 ° C.

2.6.3 Effect of incubation Time

The effect of incubation time i.e., 1 to 6 days on the cellulase production was studied.

2.6.4 Effect of Different substrate concentration

To find the suitable concentration of substrate (coir pith), production was carried out using different substrate concentrations such as 0.5, 1.5 and 2 g/ 100 ml of the production medium.

2.6.5 Effect of different Inoculum size

To find the suitable inoculum size, various concentration of inoculum size 0.5, 1, 1.5 and 2 ml/100 ml was inoculated to the production medium.

2.7 Production of cellulase enzyme using coir pith

Strain presenting cellulolytic activity was used for enzyme production assays on liquid medium. The liquid medium containing 0.14 % $(NH4)_2SO_4$, 0.6 % K_2HPO_4, 0.20 % KH_2PO_4, and 0.01% $MgSO_4$ $7H_2O$, pH 6.0 was inoculated with a 1 ml of culture. Culture were grown in 250 ml Erlenmeyers flasks with 100 ml of medium in a rotary shaker (100rpm) at 40˚C and incubated for 72 hrs. After 72 hrs the supernatant was separated by centrifugation and used to evaluate total cellulase activity

2.8 Determination of total cellulase activity:

Total cellulase activity of newly isolated cellulolytic bacterial strain was determined as described by **Mandels *et al.*, (1976)**. An aliquot of 0.5 ml of cell-free culture supernatant from each bacterial culture was taken in a clean test tube and 1 ml of Sodium citrate buffer (pH 5.8) (0.336 g of citric acid and 2.470 g of trisodium citrate was dissolved in 100ml of distilled water). At the

temperature of 50°C, one strip of Whatmann no. 1 filter paper 1.0 x 6.0 cm (~ 50 mg) was added to each tube and incubated for 1 hr. Tubes were vortexed till the filter paper settled at the bottom of the tube. After incubation, 3.0 ml of Dinitrosalicyclic acid (DNS) was added to each tube and mixed well. The glucose standards were prepared by dissolving 0.2- 5.0 mg of glucose per ml and the enzyme blank was prepared by mixing 1.0 ml citrate buffer and 0.5 ml enzyme. Blank was prepared by adding 1.5 ml sodium citrate buffer with 3.0 ml DNS. The sample mixtures, glucose standards, enzyme blank and the blank were boiled together for exactly 5 min in a vigorously boiling water bath. After boiling, the tubes were transferred to a cold water bath and 20 ml of distilled water was added, mixed by completely inverting the tube several times. The colour formed was measured against the blank at 540 nm. Cellulase activity was expressed in filter paper unit (FPU) per ml of undiluted culture filtrate. One FPU is defined as the quantity (in mg) of reducing sugar liberated in one hr under the standard assay conditions. Reducing sugar produced in one hour was calculated by comparing A 540 with that of standard curve. Exoglucanase unit was calculated using the formula, FPU/ml units ml^{-1} = mg glucose released x 0.185 was calculated **(Ghose, 1987).**

3 Results

3.1 Isolation and identification of cellulolytic bacteria:

Fifty bacterial strains were isolated from soil sample and screened for cellulase activity. On screening among fifty bacterial isolates, twelve isolates showed cellulolytic activity by forming zone formation on CMC containing agar plates. Out of twelve cellulolytic bacteria strain no SACT9 showed higher zone formation of 0.8 cm (in diameter) and cellulase activity of 0.480 (U/ml) **(Fig 1)**. Isolated cellulolytic bacterial strain SACT9 was gram negative, showed positive characteristics for motility, indole utilization, Voges proskauer, Citrate utilization, glucose fermentation, catalase, oxidase and nitrate reduction tests. Isolated cellulolytic bacteria showed similar biochemical characteristics and identified as *Pseudomonas species* **(Table.1)**.

3.2 Pretreatment of coir pith:

Coir pith was collected from agriculture lands in Samayapuram village,Trichy. The collected samples were pre-treated with acid pre-treatment and bleached. Bleached substrates colour got changed from dark brown to pale yellow.

3.3 Estimation of cellulose in the coir pith:

Cellulose was estimated using nitric/ acetic acid reagent. The un pre-treated coir pith contains 132 mg of cellulose while pretreated coir pith contains 97 mg of cellulose.

3.4 Optimization of media

3.4.1 Optimum pH:

In order to determine the optimum pH value for the bacterial enzyme, the activity of the enzyme was assayed between the pH 2 to 10 and higher cellulase activity was found at pH 6 **(Fig 2)**.

3.4.2 Optimum temperature:

The optimum temperature for cellulase production was determined by assaying cellulase activity at different temperatures (20 –80˚C). The optimum temperature was found to be 40˚C for cellulase production because isoalted strain showed cellulase activity of 0.25 (U/ml) in media containing coir-pith **(Fig 3)**.

3.4.3 Optimum Incubation:

Effect of incubation time showed varitions in cellulase production **(Fig 4) .** The isolated strain showed efficient enzyme activity at 74 hrs of incubation.

3.4.4 Effect of substrate concentration:

The production medium was inoculated with culture containing various substrate concentrations (0.5-2 g). The cellulase production was higher at 1.5 g of substrate with 0.120 (U/ml) **(Fig 5)**.

3.4.5 Effect of inoculum:

The production medium was inoculated with culture containing various inoculum concentrations (0.5-2 ml). The cellulase production was higher at 1ml of inoculum and less production in 2 ml of inoculum size **(Fig 6)**.

3.5 Enzyme production:

The production media was prepared, cellulolytic bacteria was inoculated and grown at optimum conditions kept in a rotary shaker (100 rpm). After incubation the supernatant was separated by centrifugation and used to evaluate total cellulase activity. After fermentation , at optimum conditions celllulase enzyme activity was higher of 0.960 (U/ml) by isolated cellulolytic bacteria SACT9 and confirmed as efficient cellulase enzyme producer .

Figure.1: Total cellulase activity of bacterial isolates from the soil sample

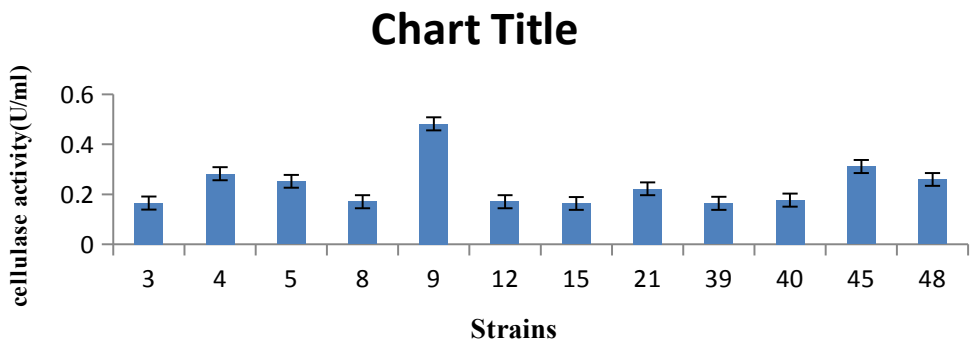

Figure.2: Effect of pH on cellulase production

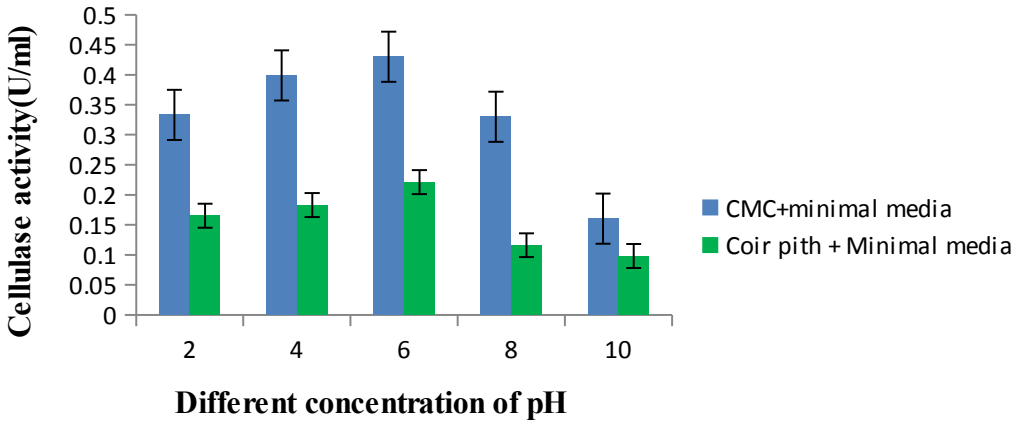

Figure.3: Effect of temperature on cellulase production

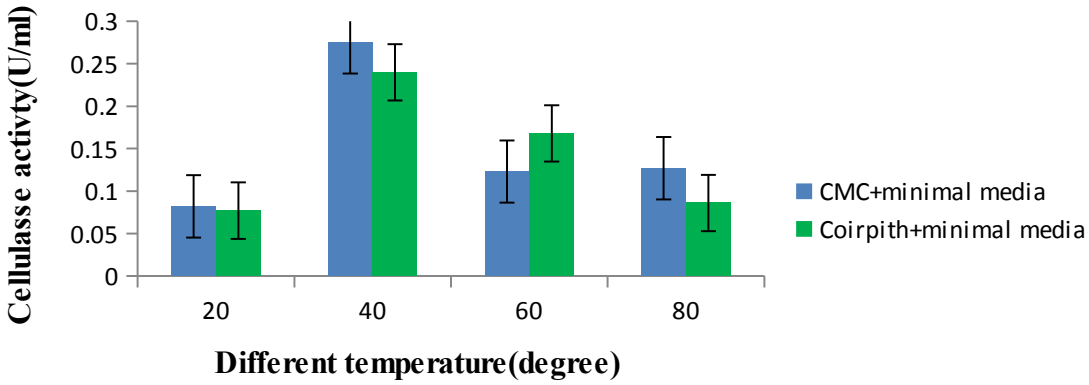

Figure.4: Effect of incubation on cellulase production

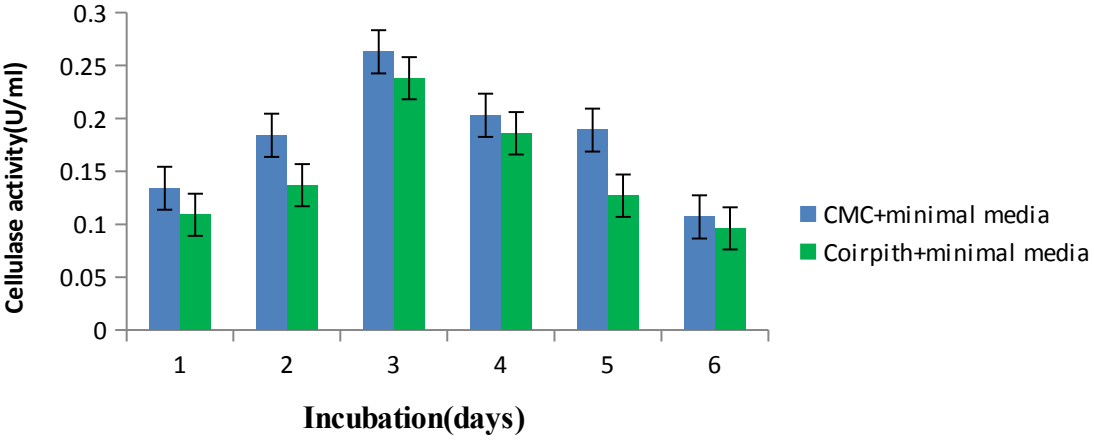

Figure.5: Effect of Substrate concentration

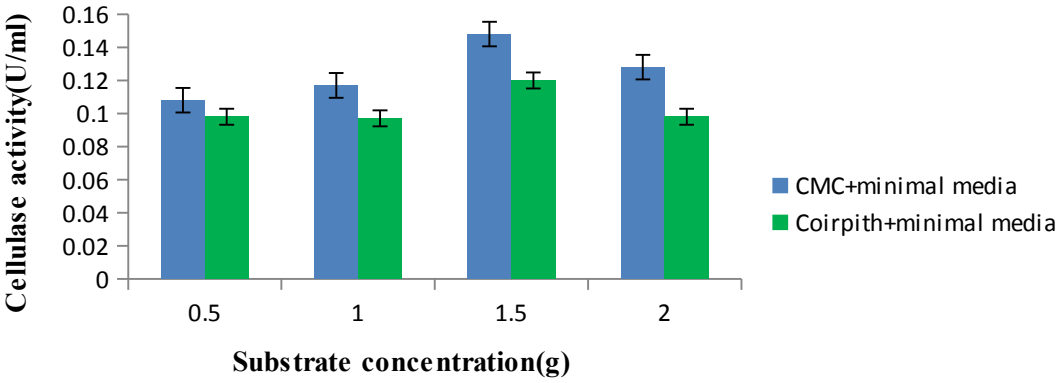

Figure.6: Effect of Inoculum

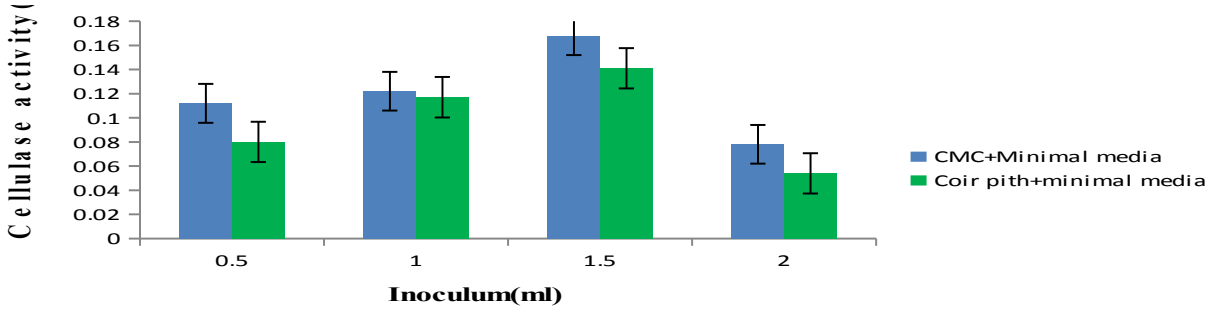

Table .1: Biochemical characteristics of isolated cellulolytic Bacteria:

S.NO	Biochemical characteristics	Results
1	Gram staining	+
2	Shape morphology	R
3	Motility	+
4	Indole utilization	+
5	Methyl red test	-
6	Voges proskauer	+
7	Citrate utilization	+
8	H_2S production	-
9	Glucose fermentation	+
10	Lactose fermentation	-
11	Sucrose fermentation	-
12	Coagulase test	+
13	Catalase test	+
14	Oxidase test	+
15	Urea utilization	-
16	Nitrate utilization	+

+ = Positive, - = Negative, R = rod

4.0 Discussion

Cellulase enzyme has a wide range of applications in food, animal feed, textile, fuel and chemical industries. Other areas of application include the paper and pulp industry, waste management, medical/pharmaceutical industry, plant protoplast production and in the treatment

of pollutants **(Mandels, 1985; Beguin and Aubert, 1993)**. Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes. Habitats that contain these substrates are the best sources in which to find these microorganisms. Screening for the isolates with cellulolytic activity revealed that the bacteria were more prolific producers of the enzyme.

Treatment of lignocellulosic materials with diluted acids can efficiently improve the enzymatic hydrolysis. Dilute-acid hydrolysis is probably the most commonly applied method among the chemical pretreatment methods. It can be used either as a pretreatment of lignocellulose for enzymatic hydrolysis, or as the actual method of hydrolyzing to fermentable sugars. The main reaction that occurs during acid pre- treatment is the hydrolysis of hemicellulose, especially xylan as glucomannan is relatively acid stable. During acid pretreatment solubilized lignin will quickly condensate and precipitate in acidic environments **(Liu and Wyman, 2003; Shevchenko *et al.,* 1999)**. The 3% HCl pre-treatment of coir pith gave better results of cellulose content compared to other pre- treatment methods.

Coir pith was selected as a source for obtaining desirable cellulose producing organisms, because it is a rich source of cellulose, So that diverse group of cellulolytic microorganisms can utilize them. Further, its wide availability, ease of processing and cost effectiveness also plays an important role for its selection. The bacterial colonies were isolated from soil sample and screened for cellulolytic activity. Fifty colonies were screened on that only one bacterial strain SACT9 possessed higher cellulase activity with higher zone formation. Then isolated bacterial strain was characterized for their morphological and biochemical characteristics. On biochemical characterization the isolated strain was identified as *Pseudomonas sp*. Media optimization is an important aspect to be considered in the development of fermentation technology. The isolated *Pseudomonas species* was inoculated in fermentation medium and production of cellulase was assayed.

Enzyme production was tested with different pH, different temperature, different carbon sources and nitrogen sources. Based on the results, the fermentation media has been designed and the production of cellulase was carried out. The culture used for inoculation in the fermentation medium must be in healthy, active state and of optimum size, possibly in the log phase, thus it will be in its high rate for substrate conversion. Cellulase is an inducible enzyme and it is affected by the nature of the substrates used for production. When Coir pith was used as substrate, the enzyme activity was observed to increase at 48h incubation. On further incubation the values were found to decrease. The enzyme activity of strain was gradually increased with

the increase in temperature and the highest cellulase activity was found to be 0.240 (U/ml) at 40 ° C. Cellulase production is increased as the pH values are increased from 4 to 6 and reached its maximum at pH of 6 with the activity of 0.221 U/ml respectively. Coir pith of 1g showed higher enzyme production with 1.5 ml inoculum. The substrates of cellulase enzyme production under mechanical shaking increased the medium aeration and permitted better contact between the substrate and the micro organisms causing significant differences in favour of the quantity of enzyme produced in agitated system. The submerged fermentation technique have been widely used in the production of cellulases and the other enzymes **(Haltrich *et al.*,1996).**The production of cellulase from coir pith in submerged fermentation under optimum conditions were performed and the enzyme production was higher with the amount of 0.960(U/ml).

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