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ISOLATION AND CHARACTERISATION OF CELLULOLYTIC ACTIVITY OF BACTERIA AND FUNGI FROM THE SOIL OF PAPER RECYCLING UNIT AT PERIYAR MANIAMMAI UNIVERSITY

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ABSTRACT

Enzymes are mostly used for the production of food and pharmaceutical products and the major source of these enzymes are microorganisms, plants, and animals. The most important group of hydrolytic enzymes is cellulase. Here we use microorganisms to produce cellulase to get high yield at low cost and the microorganisms were isolated from the soil found at the paper recycling unit. The bacteria and fungs were isolated and identified using the standard methods and the bacteria were studied for its glucose concentration and the fungs were studied for the cellulase production. Totally 6 bacteria and 8 fungus were isolated and identified. Among the bacteria only *Brucella canis*, *Enterobacter aerogenes* and *Eruvinia psidii* produced high yield of glucose and the fungus *Torula herbarum* produce high yield of cellulase. Finally, we concluded that for the production cellulase microorganism should be the good source in addition to that paper recycling unit soil would be the new and effective source for the enzymes.

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INTRODUCTION

Enzymes play a major role in most of the food and biotech industries for the production of commercial available food and pharmaceutical products (Gurung N *et al*., 2013). Among the number of industrially important enzymes cellulases is one of the essential and a group of hydrolytic enzymes capable of hydrolysing the most abundant organic polymer. The main source of the enzyme are microorganisms, plants, and animals (Sherief *et al.,2010;* Okemoto.,*et al* 2003). Instead of different source, we select microorganisms for the production of cellulase, this is due to its high yield and low production cost when compare with others (Maki M *et al*., 2009). Microbial degradation of lingo cellulosic waste and the downstream products resulting from it are accomplished by a concerted action of several enzymes, the most prominent of which are the cellulases (Sukuraman *et al.*, 2005). Our group have published number of preliminary articles (Jeyajothi Kalimuthu *et al*., 2016; S.C.G.K. Daniel *et al*., 2015; J. Kalimuthu *et al*., 2014) related to the current article. Our current study was aimed to screen the cellulolytic ability of cellulases from bacteria and fungi present at the soil in the paper recycling unit. Furthermore, the induction of enzyme synthesis and optimal condition for their activity were also determined

EXPERIMENTAL:

Materials:

The soil was collected from the paper recycling unit at Periyar Maniammai University, Vallam, Thanjavur, Tamilnadu, India (Plate - 1). The chemicals used were supplied by high media chemicals and the glass wares from borosilicate. All the materials and glass wares were completely sterilized and the entire project was carried out in a complete sterile condition in microbiology lab, department of biotechnology, Periyar Maniammai University.

Plate - 1

Recyle paper unite waste soil

Freshmut

METHODS:

Primary studies:

The bacteria was isolated by serial dilution method (Ben-David, A and Davidson, C.E. 2014) and they were characteristics using colony morphology and staining studies (Holt *et al*., 1994). Among the different staining procedure here we use standard gram staining procedure described by Todar *et al*., 2005. In addition to that we also well-found the movement of the bacteria by motility determination test (Aygan and Arikan 2007) And for the fungi, followed by the basic isolation methods the pure culture was identify by Lacto phenol cotton blue mounting method (S.C. Parija *et al*., 2003).

Biochemical tests:

After completing the basic tests, we also need to analysis various biochemical tests using various reagents to get the better identification of bacteria. Among number of biochemical tests, depend upon the bacteria we need to carry out only few of them. The commonly used biochemically tests are listed below. Catalase test (Bhattacharya. C *et al*., 2014) is carried out to check the presence of enzyme catalase in bacteria likewise methyl red test (Dubey R.C.; 2002) is to detect the presence of large concentrations of acid end products, To found the presence of acetyl methyl carbonyl vogues proskauer test (A. L. Barry and K. L. Feeney 1967)can be done, the Indole Test (Bhattacharya. C *et al*., 2014) was carried to determine the ability of organism to convert the tryptophan into indole., Nitrate reduction test (H. J. Conn and R. S. Breed 1919) is used to find the ability of the organism to reduced nitrate, Glucose broth with Durham tubes test (Hayward, A.C. 1957) is helpful for us to find the ability of organism to ferment the glucose, Citrate Utilization Test (Bhattacharya. C *et al*., 2014) is used to check the production of enzyme citrate permease by the organism, Oxidase Test (P Jurtshuk, Jr and D N McQuitty 1976) is to check that the organism contains the enzyme cytochrome oxidase, Urease Test (Bhattacharya. C *et al*., 2014) identifies that the organism have the capability to hydrolyse urea, Triple Sugar Iron Agar Test (Sulkin E.S. and Willett J.C., 1940) is carried to check that the organism have the ability to ferment sugar into acid. All these testes were carried out carefully using standard procedure to clearly characterize the organism.

Enzyme Studies:

Among the Fungi isolates *Torula herbarum* and *Verticillum sp.* were selected for enzyme studies of cellulase.

Assay for Cellulase:

Cellulase positive cultures were inoculated into modified Czepek cellulase broth and incubated for 7 days. 0.5ml of culture filtrate was mixed with 0.45ml of 1% carboxymethyl cellulase and kept at 55°C for 15 minutes. Then 0.5ml of dinitrosalicylic acid was added. The mixture was heated in boiling water bath for 5 minutes. 1ml of potassium sodium tartarate was added and made upto 5ml with distilled water and the colour intensity was measure by spectrophotometer at 540nm (Denison and Koehn, 1977). Glucose act as standard solution protein and its content was estimated by Lowry's method (Lowry's *et al.,* 1951).

Estimation of Protein:

We carried out the Lowry's assay for the estimation of protein.

Protein Calculation:

The amount of protein was calculated by the standard procedure:

Test OD/ Std. OD * Con. of Std. * 1/ Volume of sample taken

Enzyme Calculation:

The amount of enzyme presence was calculated by the standard method

Test OD/ Std. OD * Con. of Std. * 1/ Vol. of samp. * 1/ Time of incu. * 1/ mg of pro.* 1/Mol. Wt. Of STD

Glucose Estimation

Take ten test tubes and label them as blank and S1-S2 sample 1,2,3,4.Make dilution of glucose standard with concentration of 0.1,0.2,0.3,0.4,0.5 mg/ml by transferring respective amount of glucose from standard glucose solution [1mg/1ml] to S1-S5 test tubes and adjusting it to total volume of 1000 ml by added distilled water.1ml of (9500ul of sample +50ul of water) sample was taken in test tube marked as sample S1,S2,S3,S4. Add 1ml of 5% phenol solution to all the tubes. Add 1ml of 98% grade H2SO4 to each tubes. After 10 minutes, mix the content and place it in water bath at 30c for 20 minutes. Switch on spectrophotometer, set the wavelength of 490nm. First take the absorbance of blank. Take OD of all the tubes. 10gm of fresh mud was weighed and inoculated into 150ml of Nutrient broth. Kept the flask in solar for 4days at 120 rpm. At the end of 4^{th} days, 1ml of sample was taken for the glucose assay. Glucose assay was done by phenol sulphuric acid method (P. Rao, and T.N. Pattabiraman 1989).

RESULTS:

Identification of bacteria:

Six bacterial species were subjected to biochemical tests for identification. The bacteria species are *Actinobacter baumanni, Bacillus subtilis, Brucella canis, Buttiauxella agrestis, Enterobacter aerogenes*, and *Eruvinia psidii* were identified (Table 1; Plate 2 & 3).

Bacteria characters: (Plate: 2)

Acinetobacter baumanii:

Rods 0.9 – 1.6 µm in diameter and 1.5 – 2.5 µm in length become spherical in the stationary phase of growth. They commonly occur in pairs and also in chains of variable length. Cells do not form spores.

Bacillus subtilis:

Cells are rod-shaped and straight, 0.5 – 2.5 X 1.2 – 10 µm and often are arranged in pairs or chains, with rounded or squared ends. Cells stain gram positive and are motile by peritrichous flagella. Endospores are oval or sometimes round or cylindrical and are very resistant to many adverse conditions.

Brucella canis:

Cocci, coccobacilli, or short rods, 0.5 – 0.7 X 0.6 – 1.5 µm arranged singly and, less frequently, in pairs, short chains, or small groups. Stain gram negative cells are non-motile and do not produce flagella.

Buttiauxella agrestis:

Small, rod-shaped cells are motile by peritrichous flagella. Gram negative, facultatively anaerobic, chemo-organotrophic, having both a respiratory and a fermentative type of metabolism, optimal temperature is 30 - 37°C.

Enterobacter aerogens:

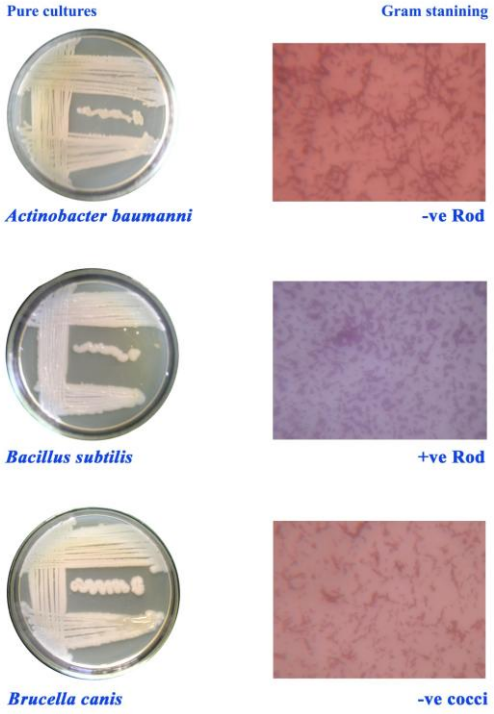
Straight rods, 0.6 – 1.0 µm wide X 1.2 – 3.0µm long, gram negative. Motile by peritrishous flagella. Facultatively anaerobic and chemoorganotrophic, having both a respiratory and a fermentative type of metabolism.

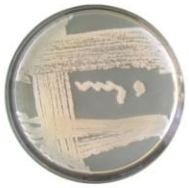
Erwinia psidii:

Straight rods, 0.5 – 1.0 X 1 – 3 µm, occur singly or pairs and sometimes in short chains. Gram negative, motile by peritrichous flagella. Facultatively anaerobic and chemo-organotrophic, having both a respiratory and a fermentative type of metabolism.

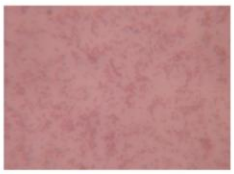
Plate - 2

Isolation of bacteria from recyle paper soil



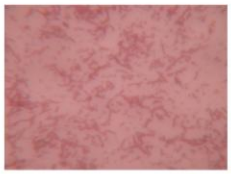


Buttiauxella agrestis



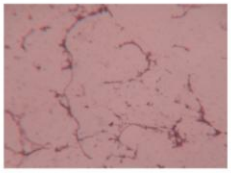
-ve cocci

Entrobacter aerogenes



-ve cocci

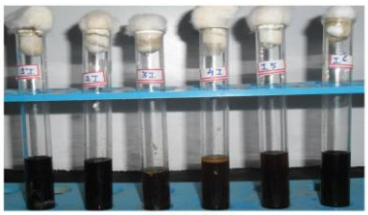
Eruvinia psidii



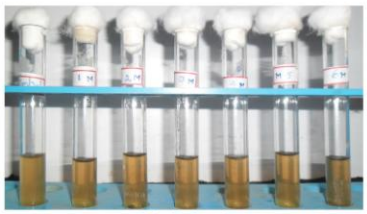
-ve cocci

Plate - 3

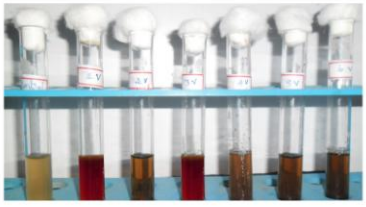
Biochemical test



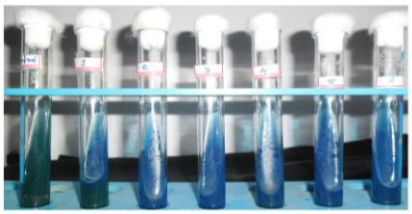
Indole Test



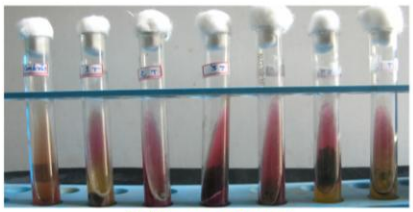
Methyl red test



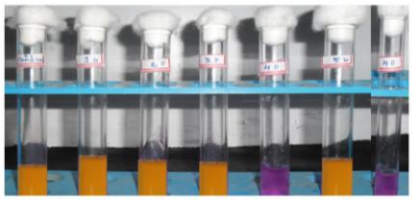
Voges Proskauer test



Citrate test

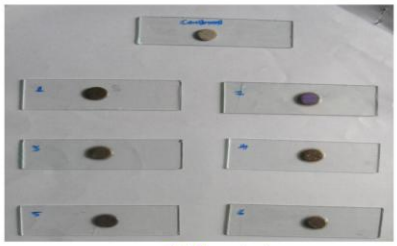


Trible sugar Iron test

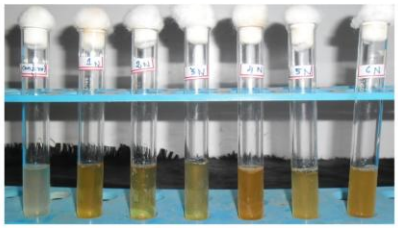


Urease test

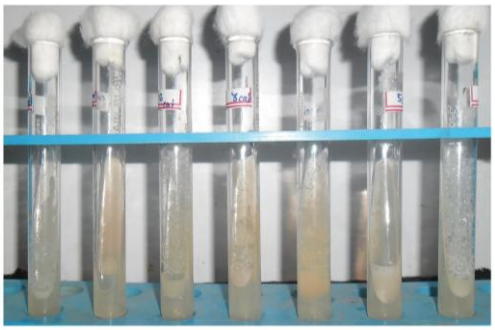
Carbohydrate Fermation test



Oxidase test



Nitrate test



Catalase test

Table 1. Biochemical test of bacterial isolates.

S. No.	Gram	Motility	Indole	M.R.	VP.	Citrate	TSI	Urease	Carbo.	Oxi.	N	Cata.
1	Gram positive rod	Motile	A	A	P	P	Yellow Alkaline	A	P	A	P	P
2	Gram negative rod and cocci	Non Motile	A	A	A	P	Yellow alkaline	A	P	P	P	P
3	Gram negative rod	Motile	A	A	P	P	Yellow pink	A	P	A	P	P
4	Gram negative rod	Motile	A	A	A	P	Alkaline pink	P	P	A	P	P
5	Gram negative rod	Motile	A	A	A	P	Pink black yellow	A	P	A	A	P
6	Gram negative rod	Non Motile	A	A	A	P	Pink black yellow	P	P	A	P	P

M. R. _ Methyl Red test
V. P. _ vogues proskauer test
Carbo. _ Carbohydrate
Oxi. – Oxidase
N – Nitrate
Cata. – Catalase
A _ Absence
P _ Presence

Glucose concentration of bacteria:

Four bacteria were selected for analysis namely *Bacillus subtilis, Brucella canis, Enterobacter aerogenes* and *Eruvinia psidii.* Glucose production in *Eruvinia psidii* (0.580) is high followed by *Enterobacter aerogenes* (0.451) *Brucella cani* (0.210) *and Bacillus subtili* (0.116) and glucose concentration of different bacteria was tabulated in table 2 and compared them in bar graph as figure 1.

Table 2. Glucose concentration of bacteria

S. No.	Name of the organisms	Glucose concentration [mg/ml]
1.	*Bacillus subtilis*	0.116
2.	*Brucella canis*	0.210
3.	*Enterobacter aerogenes*	0.451
4.	*Eruvinia psidii*	0.580

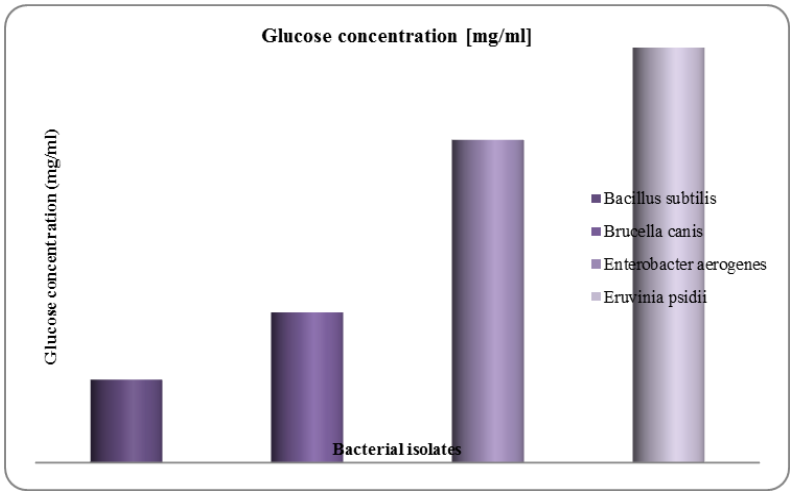


Figure 1: Glucose concentration of bacteria.

In the present investigation totally 8 fungal species were identified by using lacto phenol cotton blue method namely *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Curvularia lunata, Penicillium chrysogenum, Penicillium janthinellum, Torula herbarum, and Verticillium* sp.(Totally 8 fungus species represending 5 genera were idendified. The maximum number of fungal isolates belonging to the class Deuteromycetes). Among the 5 genera recorded, the genus Aspergillus (3 species) was dominant followed by Penicillum (2species). All other genera were represented by one species each (Plate 4).

Fungal Characters: (Plate – 4)

Aspergillus niger:

Colonies on Czapek's agar rapidly growing with abundant submerged mycelium, in some strains with more or less yellow colour in the hyphae, aerial hyphae usually scantily produced. Revere usually without colour.

Aspergillus flavus:

Colonies on Cazpek's agar widely spreading, with floccosty limited to scanty growth of a few aerial hyphae in older areas. Conidial areas ranging in color from sea. Foam velloco through ehartreuse – yellow, citron – green or lime – green to mignonette green.

Aspergillus fumigatus:

Colonies on Czapek's agar in some strains strictly purple – red, with varying amounts of tyfte aerial mycelium up to dye and with two forms, green to dark green, becoming almost black in age, spreading. Reverse and substratum, colorless to yellow.

Curvularia lunata:

Colony spreading, subgloccose, dark olive – gray, reverse bluish – black, hyphae septate and much branched, olive, 3 – 3.6µm in diameter. Conidia the phores erect, more than 100 µ long X 3.6 µ in diameter.

Pencillium chrysogenum:

Green gray velvety colonies with a heap of white mycelium at the centre. The reverse is yellow white, strong smell. The conidiophores are smooth walled and are 200 - 100µm. there are 3 to 5, metulae of 8 – 12 X 2 – 4 µm.

Penicillium janthinellum:

Colonies gelatin bluish green reverse yellow in colour. Conidiophores 30 – 40 X 2 µm arising from, creeping hyphae. Conidia globose, 2.4 - 3 µm.

Torula herbarum:

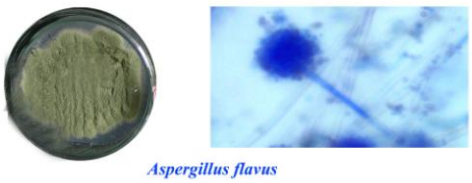
Torula sp. is commonly found indoors on cellulase containing materials. It produces type I allergies. There have been no reports of human infection. *Torula* sp. is capable of producing toxins in specific circumstances, although the effects of these toxins are not well known. This was studied by observing the colony morphology of the fungi. Here different colonies were identified on the basis of morphology.

***Verticillium* sp:**

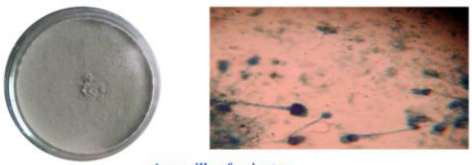
Sterile hyphae creeping, septate, branched, hyaline or lightly colored. Conidiophores erect, septate, branched. Branches of the first order whorled, opposite or alternate branches of the second order whorled, dichotomous or trichotomous on the branches of the first order.

Plate - 4

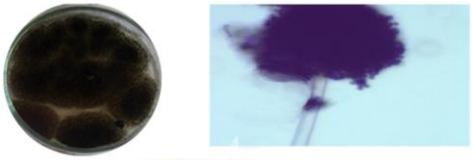
Isolation and Identification of Fungus



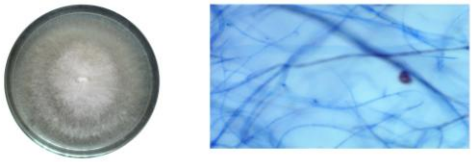
Aspergillus flavus



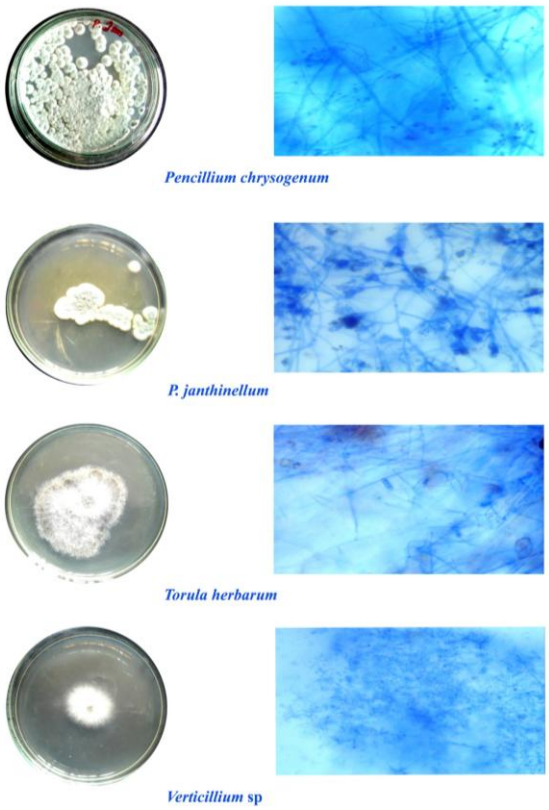
Aspergillus fumigatus



Aspergillus niger



Curvularia lunata



Cellulase activity: (Plate - 5)

The maximum level of cellulase production was recorded in *Torula herbarum* (0.022 IU/ml) at 28^{o}C minimum level of cellulase production was observed in *Verticillium* sp (0.019 IV/ml) at 28^{o}C and cellulase activity of different fungi was tabulated in table 3 and compared them in bar graph as figure 2.

Plate - 5

Cellulose production of fungus

Control ***Torula herbarum***

Control ***Verticillium* sp**

Table 3. Cellulase activity of fungi.

S.No	Organism	Enzyme activity [Iu/ml)
1.	*Torula herbarum*	0.022
2.	*Verticillium* sp	0.019

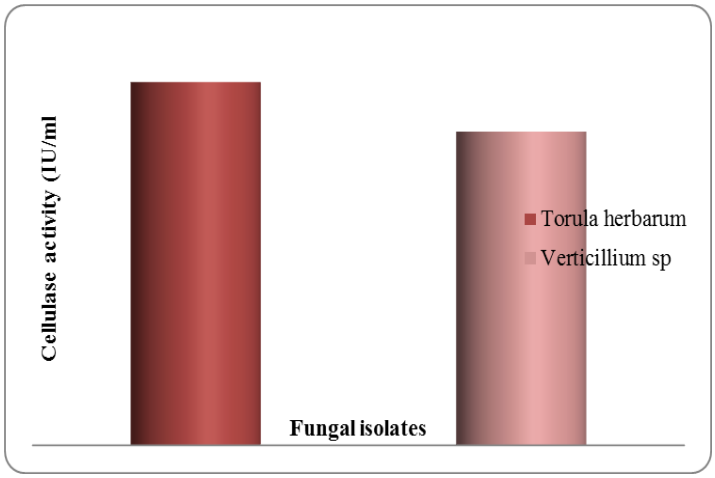


Figure 2. Cellulase activity of fungi.

CONCLUSION AND FUTURE PERSPECTIVE:

Thus study concluded that recycling environment provides impressive diversity of fungus and bacteria. Totally 6 bacteria and 8 fungus were isolated and identified. Among the bacteria only *Brucella canis, Enterobacter aerogenes* and *Eruvinia psidii* produced high yield of glucose and the fungus *Torula herbarum* produce high yield of cellulase. Therefore isolated fungus would provide a new source for the effective cellulase production. Our team published already number of research article in the purification of different enzymes especially allinase (Rathnaswamy. S *et al*., 2014) and heart proteins (Sundaramoorthy. M *et al*., 2014) and review articles in medicinal plants (Balakrishnan. P *et al*., 2015, Aruna AP *et al*., 2017, Suganthi Nagarasan, Boominathan M 2016, Suganthi Nagarasan and Boominathan M 2016). In addition to that, we also published a article to mesure the growth of fungi especially *Aspergillus terreus* (S. Subrahmanyam *et al*., 2001). Further study is essential for the separation and purification of enzyme, if the purification study give the expected results then the cellulase will commercially available at very low cost.

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