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Research Article

Extracellular Cellulase enzyme Production from *Penicillium funiculosum*

A.Nagasathya[1] , D. Sivanesan[2] and E. Annes Ali[3]

[1]Department of Zoology, Govt. Arts College for Women, Pudukkottai, Tamil Nadu, India.
[2]Nova Southeastern University, Florida, USA.
[3]Department of Microbiology, J.J. College of Arts and Science, Pudukkottai, Tamil Nadu, India.
***Corresponding author e-mail:** *dranagasathya@gmai.com*

Abstract

In the present study *Penicillium funiculosum* was isolated from from mangrove habitat of Muthukuda situated near Meemisal, pudukkottai district, Tamil Nadu and we have studied its enzyme activity on different natural wastes (bagasse, rice husk and coir pith) by altering the parameters like, temperature, pH and incubation periods. Among these substrates, rice husk was found to be more effectively degraded by the organism and hence, can be the best source of enzyme. The organism was exposed to UV radiation and the mutational effect on enzyme activity was also studied.

Keywords: *Penicillium funiculosum* ; different natural wastes; temperature, pH and incubation periods; mutational effect on enzyme activity.

Introduction

Cellulases the enzyme which hydrolyze β 1,4 linkages of cellulosic substrates find wide application in foods, textiles and pharmaceuticals. Cellulases, when supplemented with other enzymes, hydrolyze tissues of various plants to release intra cellular material such as oil, protein, starch, etc.

Although a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell-free enzymes capable of completely hydrolyzing crystalline cellulose *in vitro*. Fungi are the main cellulase producing microorganisms though a few bacteria and actinomycetes have also been recently reported to yield cellulase activity. Microorganisms of the genera *Trichoderma* and *Aspergillus* are though to be cellulase producers, crude enzyme produced by these microorganisms are commercially available for agricultural use. Microorganisms of the genus *Trichderma* produce relatively large quantities of endo β glucanase and exo β glulcanase but only low levels of β glucosidase. While those of the genus *Aspergillus* produce relatively large quantities of endo β glucanase and β glucosidase with low levels of exo β glucanase production.

Hundreds of species of fungi and bacteria are able to degrade cellulose. This organism includes aerobes, anaerobes, measophiles and thermophiles. They are both widespread and abundant in the natural environment. However although many microorganisms can grow on cellulose or produce

enzyme that can degrade amorphous cellulose, relatively few produces the entire complement of extra cellular cellulases able to degrade crystalline cellulose *in vitro*. Among the latter organism the most extensively studied sources of cellulolytic enzymes have been the fungi *Trichoderma* and *Phanerochate* and the bacteria C*ellulomonas* and *Clostridium thermocellum.*

Cellulolytic enzyme systems can be produced by a number of different microorganisms such as aerobic and anaerobic bacteria (Gilkes *et al*.,1991) white rot fungi (Uzcategui *et al*.,1991 Thompson *et al*., 1998) anaerobic fungi (Barichevic and Calza, 1990) and soft rot fungi (Kubichek *et al.,* 1990).

Desai and Betrabet (1972) reported that *Penicillium funiculosum* isolated from defoliated fabrics and maintained on mineral agar slopes with filler paper strips as sole source of carbon was used for the production of cellulase. Mandels (1974) reported that the *Trichoderma* cellulase system is most effective in hydrolyzing the cellulosic substrates. Sternberg *et al*., (1977); Allen and Sternberg (1980) reported that the efficiency of *Trichoderma* cellulase system can be enhanced by supplementation of D glucosidase.

The present investigation was initiated to study about the activity of cellulase enzyme with the following objectives To screen cellulase producing fungi from mangrove soil samples collected from Muthukuda.Pudukkottai District, Tamil Nadu. To observe the enzyme activity in various parameters like pH temperature, incubation periods, natural waste (Bagasse, Rice husk, Coir pith). To compare the enzyme activity in wild and mutant type of *Penicillium funiculosum.*

Materials and Methods

Sample collection

The soil sample was collected from mangrove habitat of Muthukuda situated near Meemisal, pudukkottai district, Tamil Nadu. The salinity of the soil ranging from 40 ppt to 165 ppt.

Isolation of fungi

The soil sample collected from sampling spot was processed using serial dilutions and plating methods.

Screening of Cellulase production

The colonies observed on the PDA and RBA plates were transferred on to the growth medium amended with cellulose as sole carbon source. The growth medium used was as Modified Park's agar.The clear zone observed around the colony was the indication cellulase production.

Substrates used

In the present study, the waste materials like bagasse, rice husk and coir pith used as the carbon source in the fermentation medium. The coconut palm is known as 'kalpa vriksha' (tree of heaven) on account of its utilization and desirable features. Every part of tree is beneficial for some economic purpose. The husk of the mature coconut consist of numerous fibers known as coir fiber. Coir pith is the binding material of this. Extraction of one kilo coir fiber generate two kilos of coil pith which is considered as waste of waste. It is a fluffy light lignocelluslose material which gets liberated in the form of dust during fiber extractions.

The waste material like coir pith, bagasse, rice husk were collected and used as carbon source instead of cellulose in the fermentation medium. These substrates are good source of cellulose (Plate 5). .

Fermentation

The fungi was inoculated into the fermentation medium modified Czapek's cellulose medium and incubated at room temperature for ten days and enzyme activity and protein production was determined.

Effect of various parameters on cellulase activity

Effect of pH on cellulase activity

The fungi was inoculated in to the fermentation medium containing the substrate coir pith, bagasse and rice husk used as carbon source. The pH of the medium was adjusted in to 5,7 and 9 and incubated and the enzyme activity and protein production was determined.

Effect of Temperature on cellulase activity

The fungi was inoculated in to fermentation medium and incubated at different temperature like 30, 40 and 50 and the enzyme activity and protein production was determined.

Effect of incubation period on cellulase activity

The fungi was inoculated into the fermentation medium and incubated at different periods like. 3, 6 and 9 days and the enzyme activity and protein productions were determined.

Mutational studies

The fungi was subjected to mutation by UV for 5 minutes and the mutated fungi was inoculated into the fermentation medium which contain the carbon source rice husk, bagasse and coir pith instead of cellulose.

The mutated strain was inoculated in to fermentation medium which contain the rice husk bagasse and coir pith incubated at different pH, temperature and incubation periods and the enzyme activity and protein production were also studied .

Determination of enzyme activity

In the cell free filtrate of each culture medium the extra cellular cellulase activity was determined as follows. One ml of the cell free filtrate was mixed with 9 ml of one percent carboxy methyl cellulose in 55 mM citrate buffer (pH5) for 30 min at 40 C. At the end of the reaction time the reducing sugar liberated was determined (Plate .6).

Estimation of reducing sugar by Dinitro salicylic acid Method

1 ml of the sample was taken and mixed with one ml of DNS regent. The contents were heated in a boiling water bath for 5 minutes. When the contents of the tubes were still warm, add 1 ml of 40% Rochelle salt solution was added. After cooling the intensity of dark red colour was read at 540 nm. Standard was prepared using glucose and a graph was plotted.

Protein estimation by Lowry's method

1ml of the sample was pipetted out and the total volume made to 4 ml with distilled water. To each tube 5.5 ml of the alkaline mix (Reagent C) is pipetted out, mixed well and allowed to stand at room temperature for 10 to 15 minutes. 0.5 ml of the regent D (Folin-Ciocalteau) the reagent is pipetted out in to each tube mixing rapidly after each addition. The tubes are left as such for 30 minutes and the blue colour formed is measured at 650nm. A proper blank without the protein is used. The standard graph is drawn and the protein was determined.

Estimation of cellulase activity

One unit of cellulase enzyme is defined as the amount of enzyme that liberates 1 g of glucose from carboxy methyl cellulose.

Specific activity

Specific activity was determined by the following formula

$$\text{Specific activity} = \frac{\text{Cellulase activity (mg/ml)}}{\text{Soluble protein (mg/ml)}}$$

Results and Discussion

In the present investigation, different colonies of *Aspergillus* sp. and *Penicillium* sp. were isolated from mangrove environment, Muthukuda situated near Meemisal, pudukkottai (dist), Tamilnadu (Plate .1.&2). Among these *Penicillium funiculosum* was isolated, identified and analysed for cellulase

enzyme activity confirmed by screening methods. From the isolated fungi, enzyme activity and specific activity were also studied.

General description of *Penicillium funiculosum*

Penicillium funiculosum was identified using "A Manual of soil fungi**",** Joseph C. Gilman (1956).

***Penicillium funiculosum* Thom**

Colonies deep green, broadly spreading, surface closely floccose; reverse and medium red or purple to almost black. Conidiophores arise laterally from aerial hyphae. Conidia elliptical, green, smooth.

Screening for Cellulase enzyme

After sufficient incubation periods, clearance of zone was observed around the inoculated fungi *Penicillium funiculosum* in modified Park's agar . Based on the ratio of visible growth to that of Modified Park's agar clearing zone, the best cellulose producers can be selected, because Modified Park's agar medium contains Carboxy Methyl Cellulose and was degraded by *Penicillium funiculosum* due to the production of cellulolytic enzymes. This was well agreed with previous reports which states that among all the test isolates, *Penicillium funiculosum* was found to be a highly efficient cellulase producer .

Cellulase activity in Fermentation medium (Modified Czapek's Cellulose Medium)

At different period of 10 days incubation, during the growth of *Penicillium funiculosum* the cellulase activity and specific activity on Modified Czapek's Cellulose Medium were determined.The obtained results, the reducing sugar, cellulase activity, soluble protein and specific activity were calculated and tabulated (Table 1). In 3 days of incubation, Reducing sugar, Enzyme activity, Soluble protein and specific activity was found to be 28 g/ml followed by 6 days 46 mg/ml and by 9days 96 g/ml respectively. Among the 10 days of incubation, the maximum cellulase enzyme activity and specific activity were found to be 96 g/ml,2.4 respectively, on 9th day when compared with 3rd day and 6th day.

Masry (2002) reported that the cellulase activity was maximum in Modified Czepek's Cellulose Medium, inoculated with *Penicillium funiculosum* in ten days of incubation periods. This is in accordance with our results.

Effect of pH on cellulase activity of *Penicillium funiculosum* (wild type) on Bagasse, Coir pith and Rice husk:-

The Modified Czapek's cellulose medium containing Bagasse, Coir pith and Rice husk substrates were used as carbon source, instead of cellulose and prepared on different pH levels i.e. 5,7 and 9. the samples were incubated for different periods (3,6,9 days) and reducing sugar, cellulase activity, Soluble protein and Specific activity were calculated and tabulated (Table 2,.a,b,c and Figure 2. a,b,c). In bagasse, the cellulase activity was found to be maximum 113 mg/ml on 9 days of incubation in the pH range of 9.

The cellulase activity and specific activity on Rice husk inoculated with *Penicillium funiculosum* showed highest value of 135 mg/ml on 9 days incubation in the pH range of 9. In coir pith sample inoculated with *Penicillium funiculosum* , the cellulose activity was found to be maximum of 117 mg/ml on 9 days of incubation period in the pH range of 9. Among the 3 samples, the maximum cellulase activity was found in rice husk samples on 9 days of incubation period in the pH range of 9. These results was correlated with previous reports of Brajeshori and Koljam *et al.,* (2000), who studied the wide range of pH and pH stability of cellulase enzyme in cellulosic waste substrates.

Effect of temperature on cellulase activity of *Penicillium funiculosum* on Bagasse, Rice husk and Coir pith

Rice husk, Coir pith and Bagasse containing fermentation medium were prepared instead of cellulose and all these samples were incubated at different temperature (30, 40 and 50ºC). From the

Table. 1 Cellulase activity in Fermentation medium

Incubation	Reducing sugar	Enzyme activity	Soluble Protein	Specific Activity
3 Days	30	28	18	1.55
6 Days	49	46	27	1.70
9 Days	103	96	40	2.40

Figure.1 Cellulase activity in Fermentation medium

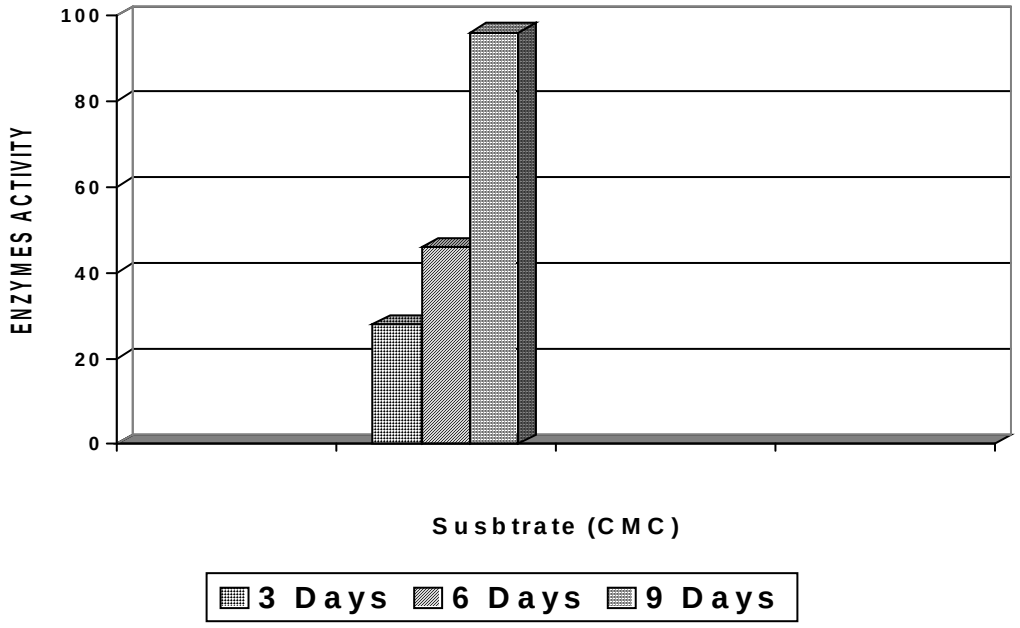


Table.2 (a) Effect of pH on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on three day incubation.

Substrate	5				7				9			
	R.S	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**
Bagasse	30	27	21	1.28	31	28	21	1.13	41	37	19	1.05
Rice husk	32	29	55	0.53	30	27	56	0.48	35	32	47	0.68
Coir Pith	27	24	30	0.80	22	19	47	0.40	31	28	56	0.50

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure.2 (a) Effect of pH on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on three day incubation.

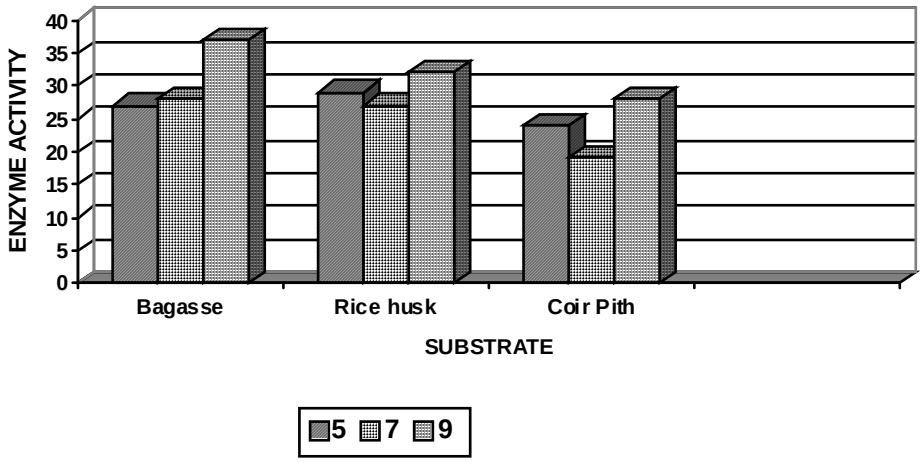


Table. 2 (b) Effect of pH on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on six day incubation.

Substrate	5				7				9			
	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A
Bagasse	57	53	21	2.52	53	49	30	1.63	55	51	42	1.21
Rice husk	65	60	57	1.05	62	58	64	0.91	67	62	56	1.10
Coir Pith	54	50	53	0.94	53	49	56	0.87	56	52	65	0.80

R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure .2 (b) Effect of pH on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on six day incubation.

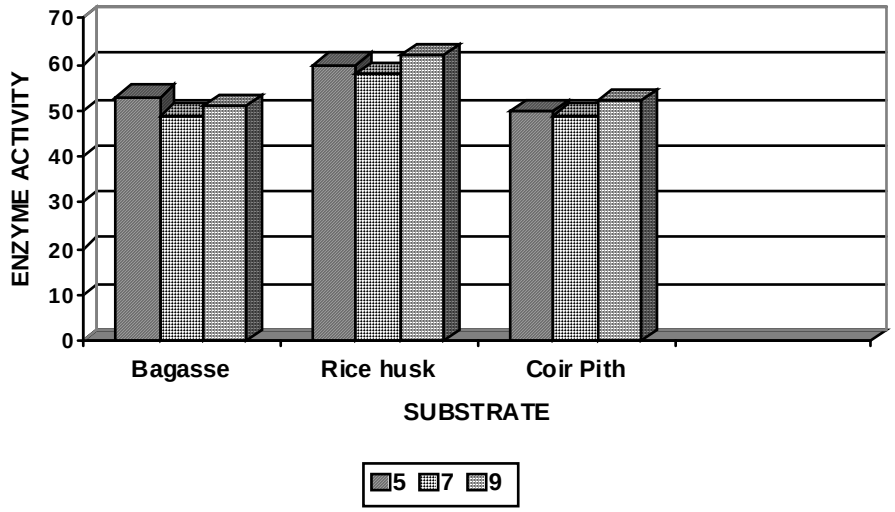


Table. 2 (c) Effect of pH on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on nine day incubation.

Substrate	5				7				9			
	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A
Bagasse	107	103	28	4.21	115	110	43	2.55	121	118	45	2.51
Rice husk	131	122	86	1.41	141	134	71	1.90	141	135	96	1.40
Coir Pith	98	92	50	1.84	117	109	59	1.84	120	117	67	1.74

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure.2 (c) Effect of pH on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on nine day incubation.

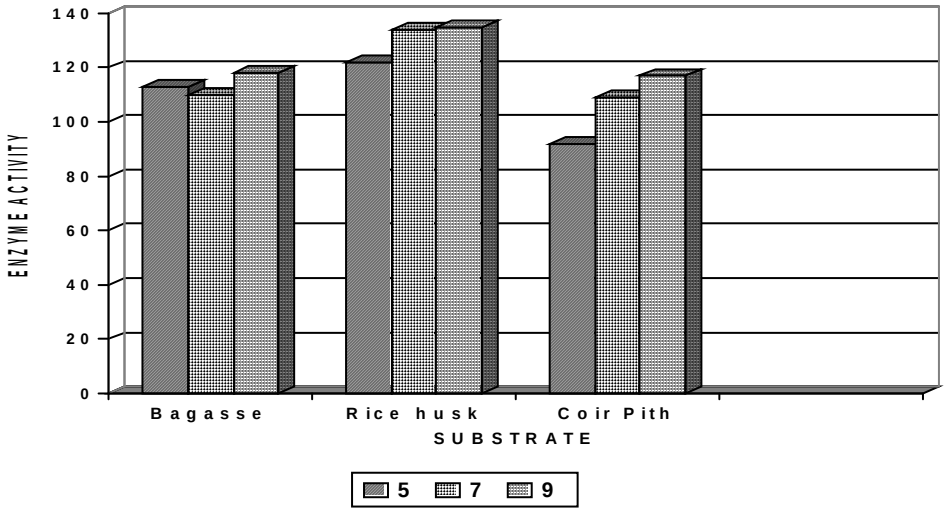


Table. 3 (a) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on three day incubation.

Substrate	30				40				50			
	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A
Bagasse	31	28	22	1.27	38	35	17	2.05	23	19	12	1.58
Rice husk	30	27	57	0.47	34	31	65	0.47	17	16	20	0.80
Coir Pith	22	19	46	0.41	33	30	62	0.48	9	8	18	0.44

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure. 3 (a) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on three day incubation.

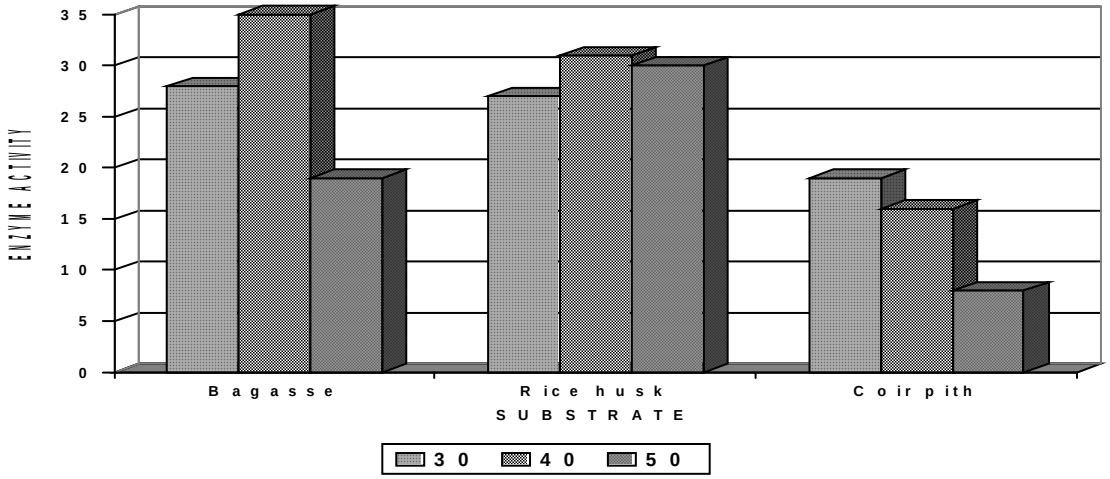


Table .3 (b) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on six day incubation.

Substrate	30				40				50			
	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A
Bagasse	53	49	23	2.13	55	50	22	2.27	28	25	18	1.38
Rice husk	62	57	65	0.87	61	56	73	0.76	19	17	26	0.65
Coir Pith	53	49	55	0.89	56	51	65	0.78	25	23	27	0.85

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure. 3 (b) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Wild type) on Bagasse, Coir pith and Rice husk on six day incubation**.**

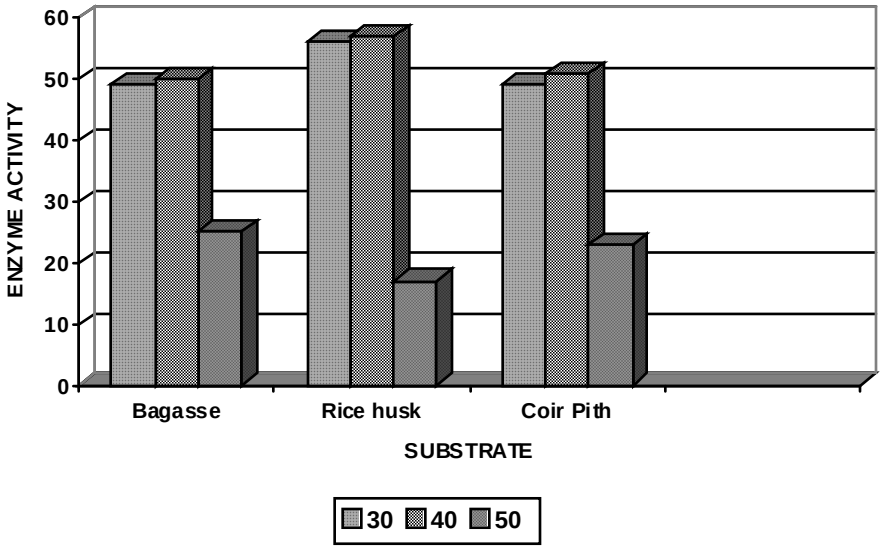


Table. 3 (c) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Wild Type) on Bagasse, Coir pith and Rice husk on nine day incubation.

	30				40				50			
Substrate	**R.S**	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**
Bagasse	98	91	42	2.16	115	100	42	2.38	39	35	20	1.75
Rice husk	138	130	71	1.83	142	135	91	1.48	62	58	35	1.65
Coir Pith	117	109	59	1.81	138	129	71	1.84	58	54	28	1.02

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity.

Figure. 3 (c) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Wild Type) on Bagasse, Coir pith and Rice husk on nine day incubation.

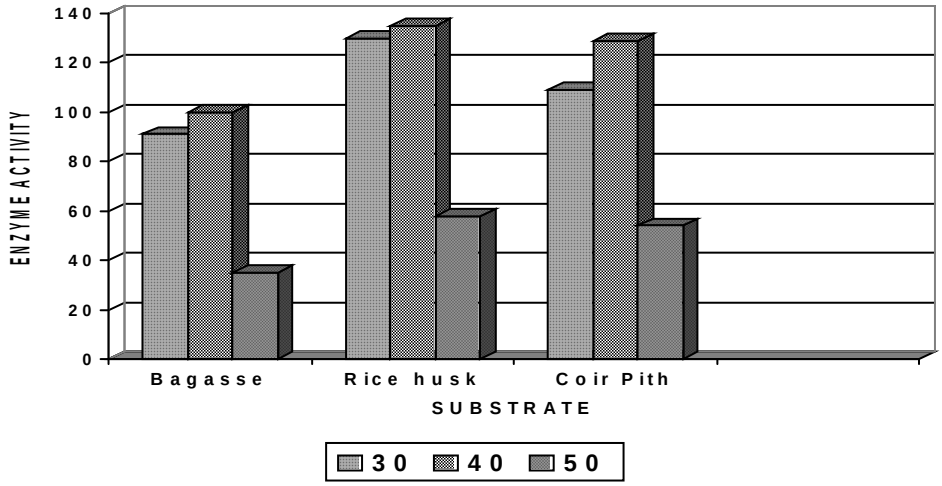


Table. 4 (a) Effect of pH on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on three day incubation.

Substrate	**5**				**7**				**9**			
	R.S	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**
Bagasse	37	34	24	1.42	38	35	17	2.05	46	42	20	2.1
Rice usk	34	31	46	0.67	34	31	66	0.47	37	34	51	0.66
Coir Pith	34	31	31	1.1	33	30	60	0.50	34	32	62	0.48

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure. 4 (a)Effect of pH on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on three day incubation.

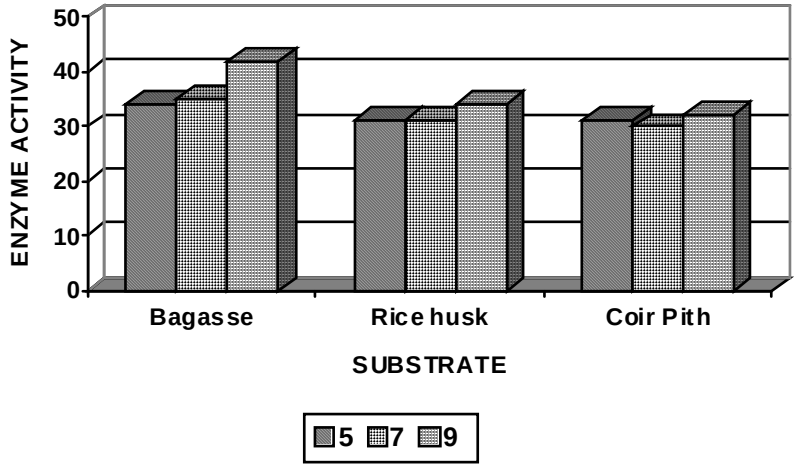


Table .4(b) Effect of pH on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on six day incubation.

Substrate	5				7				9			
	R.S	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**
Bagasse	61	54	34	1.7	55	50	22	2.27	60	58	28	1.93
Rice husk	63	58	50	1.16	61	56	73	0.76	68	62	54	1.15
Coir Pith	62	57	56	1.01	56	51	65	0.78	60	58	67	0.82

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure. 4 (b) Effect of pH on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on six day incubation.

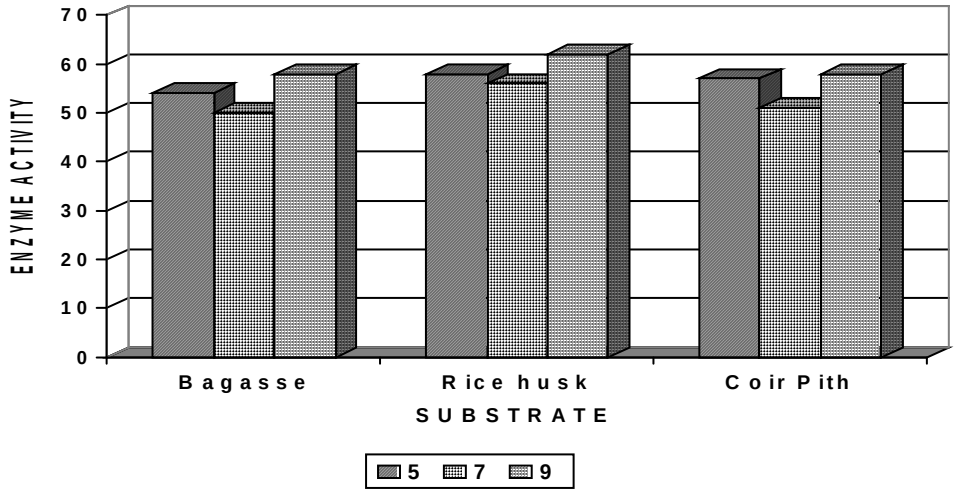


Table. 4 (c) Effect of pH on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on nine day incubation.

Substrate	5				7				9			
	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A
Bagasse	125	119	62	2.06	123	115	44	2.61	138	128	51	2.33
Rice husk	139	129	90	1.43	147	136	80	1.46	144	137	92	1.47
Coir Pith	111	104	67	1.55	126	117	71	1.64	138	128	73	1.75

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure. 4 (c) Effect of pH on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on nine day incubation.

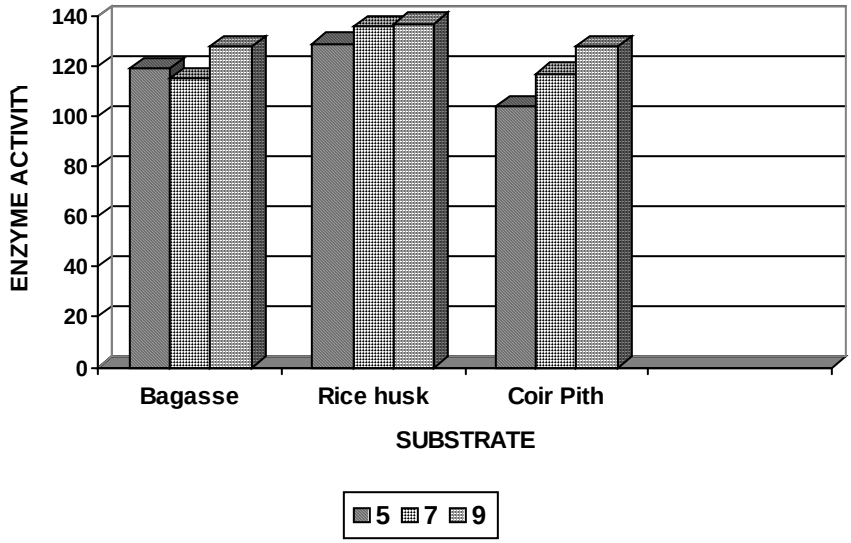


Table. 5 (a) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on three day incubation

Substrate	30				40				50			
	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A
Bagasse	41	38	19	2.0	46	42	20	2.1	20	18	10	1.8
Rice husk	35	32	46	0.69	37	34	54	0.63	15	14	27	0.52
Coir Pith	31	28	55	0.51	33	30	60	0.50	14	13	24	0.54

*R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure. 5 (a) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on three day incubation

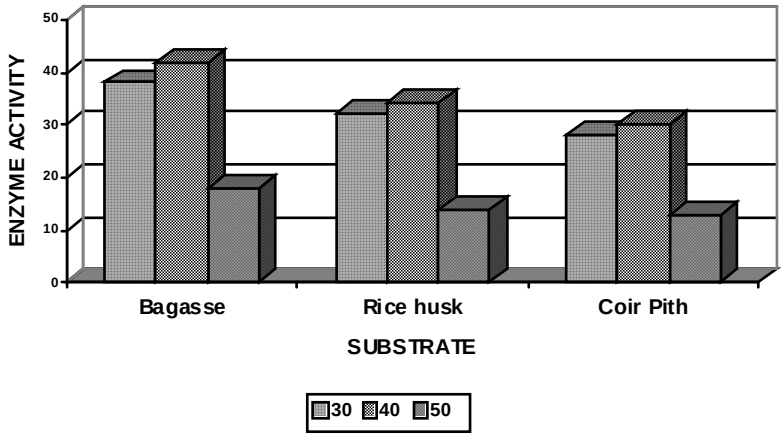


Table. 5 (b) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on six day incubation.

Substrate	30				40				50			
	R.S	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**	**R.S**	**E.A**	**S.P**	**S.A**
Bagasse	55	50	42	1.1	58	54	28	1.93	26	24	13	1.85
Rice husk	67	61	56	1.08	68	62	54	1.15	19	17	32	0.53
Coir Pith	56	50	65	0.77	60	55	80	0.69	23	21	26	0.81

***R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity**

Figure. 5 (b)Effect of temperature on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on six day incubation

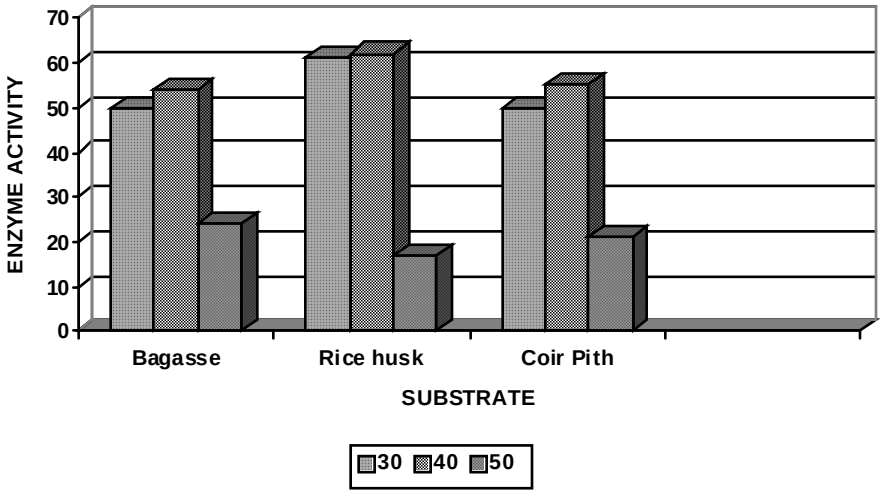
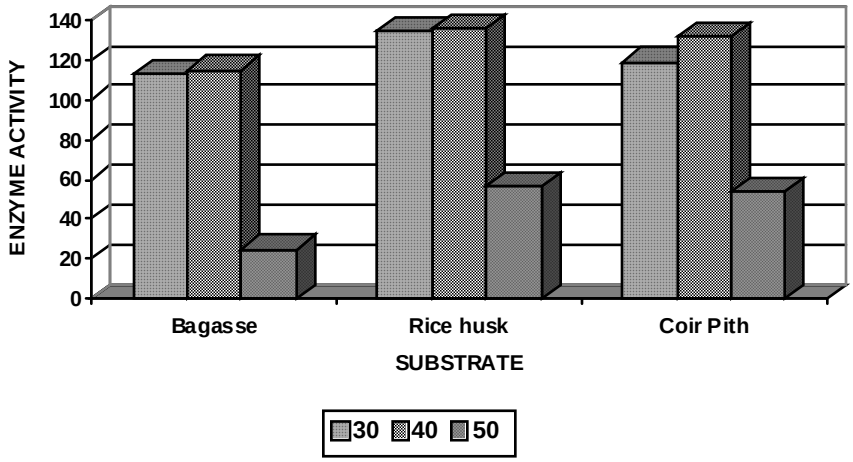


Table. 5 (c) Effect of temperature on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk on nine day incubation.

Substrate	30				40				50			
	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A	R.S	E.A	S.P	S.A
Bagasse	120	113	45	2.51	121	115	40	2.87	27	25	15	1.66
Rice husk	141	134	92	1.46	142	136	98	1.38	62	57	36	1.58
Coir Pith	127	119	69	1.72	140	132	70	1.88	61	54	31	1.74

*****R.S = Reducing sugar; E.A = Enzyme activity; S.P = Soluble protein; S.A =Specific Activity

Figure. 5 (c) Effect of temperature on cellulase activity of Penicillium funiculosum (Mutant type) on Bagasse, Coir pith and Rice husk on nine day incubation**.**



samples, Reducing sugar, Cellulase activity, Soluble protein and Specific activity were calculated and tabulated (Tables 3.a,b,c and Figure 3. a,b,c) on 3,6 and 9 days of incubation period.

The fermentation medium containing Rice husk showed the maximum Cellulase activity of 135mg/ml on 9 days of incubation period at temperature 40ºC. The maximum Cellulase activity on Bagasse was found to be 100 mg/ml on 9 days of incubation periods at temperature 40ºC.

In the fermentation medium containing Coir pith, the highest values of Cellulase activity was recorded as 129mg/ml on 9 days of incubation period at 40ºC. Among the 3 samples, the maximum Cellulase activity was exhibited in rice husk samples at 40ºC on 9 days of incubation

Mutational Studies

Effect of pH on cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk

The mutated strain of *Penicillium funiculosum* was inoculated onto fermentation medium containing Rice husk, Bagasse and coir pith samples. The Reducing sugar, Cellulase activity, Soluble protein and specific activity was determined by assay methods and tabulated (Tables 4,.a,b,c and Figures 4. a,b,c).

In mutational studies, at different pH levels the cellulase activity was found to be maximum of 119 mg/ml, 136mg/ml,128mg/ml in samples of bagasse, rice husk and coir pith at pH 9 and 9 days incubation period respectively. Among the sample rice husk showed the highest cellulase activity in pH of 9 and 9 days of incubation period.

Effect of Temp cellulase activity of *Penicillium funiculosum* (Mutant type) on Bagasse, Coir pith and Rice husk

Penicillium funiculosum mutant strain was inoculated in fermentation medium containing Rice husk, Bagasse and Coir pith instead of cellulose. From these samples, Reducing sugar, Cellulase activity, Soluble protein and Specific activity were determined calculated and tabulated. (Tables 5,.a,b,c and Figures 5. a,b,c).

At different temperature ranges in the mutated strain **of** *Penicillium funiculosum*, highest Cellulase activity was recorded at 40ºC.. the cellulase activity was of 136 115,132 mg/ml in rice husk, bagasse and coir pith samples respectively on 9 days of incubation .Among these samples rice husk shown the highest cellulase activity at 40ºC on 9 days of incubation.

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