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Influence of culture conditions on cellulase production by *sclerotium rolfsii* sacc

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Abstract
The purpose of this study was to determine the influence of culture media, incubation period, temperature and pH on the cellulase (cx) enzyme production by *Sclerotium rolfsii*. Activity of cellulase enzyme was assayed by viscometric method.

In order to select the best suitable medium, the production of cellulase enzyme was estimated by growing the *Sclerotium rolfsii* in ten different culture media for 9 days at 30º C temperature. Among ten culture media, Asthana and Hawker's, Czapek's, Glucose-dox and Glucose-nitrate broth media have not favoured the production of cellulase enzyme as in the culture filtrate of these four media, no trace of cellulase enzyme have been detected. While in other remaining six media (i.e. Basal mucor, Brown's, Dextrose- asparagine phosphate, Elliot's, Fernando's and potato dextrose) *Sclerotium rolfsii* was able to produce cellulase enzyme. Amongst these six media, potato dextrose broth medium was found to be the best for the production of cellulase enzyme. For further study, potato dextrose broth medium was selected for cellulase enzyme production.

The production of cellulase enzyme has been studied at different incubation period. The *Sclerotium rolfsii* was cultured at 30ºC on potato-dextrose broth medium for 3, 6, 9, 12, 15 and 18 days. It was found that *Sclerotium rolfsii* has a capability of producing cellulase enzyme within short incubation period i.e. 3 days incubation. Production of cellulase enzyme, gradually increased with increasing the length of incubation period up to 9 days and further increase in length of incubation up to 18 days, did not show any effect on the production of cellulase. The nine days incubation has found to be the best incubation period for the maximum production of cellulase enzyme.

The influence of different temperatures on the production of cellulase enzyme was also investigated by growing the *Sclerotium rolfsii* at various temperatures viz., 15º, 20º, 25º, 30º, 35º, 40º and 45ºC. The *Sclerotium rolfsii* was able to produce the cellulase enzyme at wide range of temperature, ranging from 15º to 35ºC. The 30ºC temperature was found to be optimum for the maximum production of cellulase enzyme.

The effect of different hydrogen ion concentrations (pH), viz., pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were also tried on the production of cellulase enzyme. The isolated pathogen *Sclerotium rolfsii* able to synthesized cellulase enzyme on a wide range of pH, i.e., from pH 3.0 to 9.0. Extremely acedic pH (i.e., at pH 3.0), the activity was found to be less which increased gradually with increase in pH up to pH 5.0. Further increasing the pH, has no effect on the production of cellulase enzyme. The pH 5.0 was found to be optimum for the maximum production of cellulase enzyme.

Keywords: *Sclerotium rolfsii*, cellulase, culture media, incubation period, temperature, pH

1. Introduction

Sclerotium rolfsii Sacc., is a soil-borne plant pathogen that commonly occurs in the tropics, sub-tropics and other warm temperate regions of the world causing root-rot, stem-rot, wilt and foot-rot on more than 500 plant species including almost all the agricultural and horticultural crops (Aycock, 1966; Domsch *et al.* 1980; Farr *et al.* 1989). It is the most destructive and common pathogen of *Solanum melongena* plants causing foot-rot disease in fields of Tikamgarh District of Madhya Pradesh (Chaurasia, 2000; Chaurasia *et al.* 2014b).

Plant biomass is made up of mostly polysaccharide in the biosphere is cellulose (Narasimha *et al.* 2006) and it is the major polysaccharide found in the plant cell wall giving the structural rigidity and strength to plants. Cellulose is an unbrached polymer composed of β – 1, 4-glucose units linked by a β-1, 4-D-glucosidic bonds

A number of plant pathogenic organisms are capable of producing multiple groups of enzymes, called cellulase that act to hydrolyze the β-1, 4-D-glycosidic bonds within the cellulose molecules (Riou *et al.* 1991; Akiba *et al.* 1995; Zaldivar *et al.* 2001; Moreira *et al.* 2005). According to the model (Figure 1) giving by Erikssion, 1969 and a proposal made by

Wood *et al.* 1989 suggest that cellulose is degraded by the synergistic action of three types of enzymes namely endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β – glucosidase (EC 3.2.1.21). According to this view endo-β-glucanase (cx) hydrolyze accessible intramolecular β – 1, 4 – glucosidic bonds of cellulose chains randomly to produce new chain ends. Exo-β-glucanase or cellobiohydrolase (C_1) progressively cleaves cellulose chains from one end to release soluble cellobiose or glucose . β – glucosidase further degrades cellobiose to release glucose.

In this way, the enzyme cellulases helps in the degradation of cellulose and after enzymatic action, the glucose yielded as final product. The glucose is preferred by a majority of the fungi and used as food to fulfill the carbon requirement and energy source for growth and other metabolic activities.

Cellulases have enormous potential in industries and are used in food, beverages, textile, laundry, paper and pulp industries etc.

The production of cellulase enzyme, and degradation of cellulose by several fungi has been studied by many workers like Bateman, 1969; Sadana *et al.* 1979; Gayal and Khanadeparkur, 1998; Zaldivar *et al.* 2001; Moreira *et al.* 2005; Narasimha *et al.* 2006; Morozova *et al.* 2010; Onofre *et al.* 2013; Chaurasia *et al.* 2013a; Nagasathya *et al.* 2014. Production of cellulase enzymes by several pathogenic fungi and its role in the development of diseases has also been reported (Amadioha, 1993; Sharma, 2000; Acosta-Rodriquez *et al.* 2005; Okunow *et al.* 2010; Sarkar *et al.* 2011; Mukharjee *et al.* 2011; Hussain *et al.* 2012; Moussa and Tharwat *et al.* 2013; Chaurasia *et al.* 2013a; Chaurasia *et al.* 2013b; Chaurasia *et al.* 2013c; Sahid *et al.* 2014; Chaurasia *et al.* 2014 a).

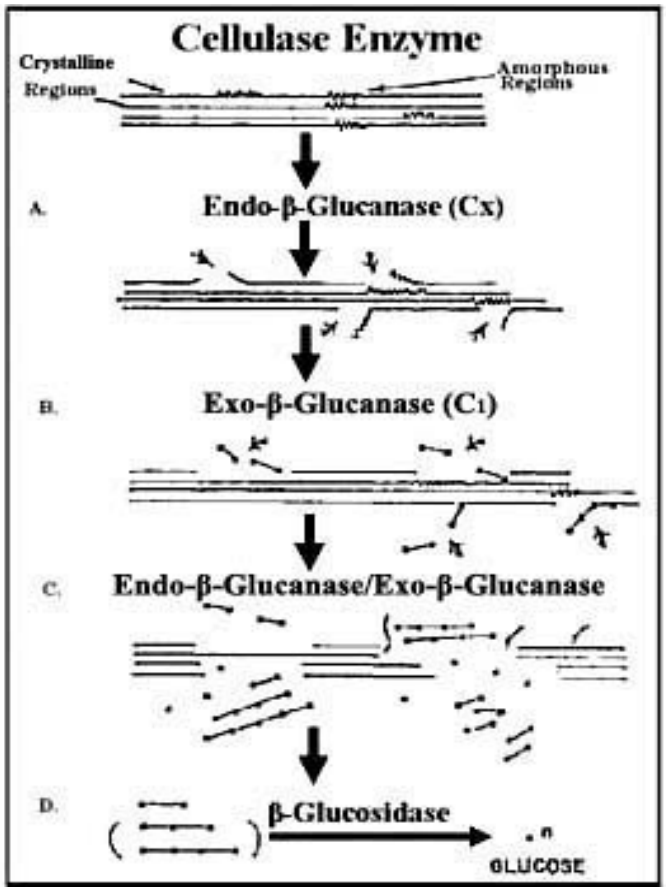

Fig 1: Schematic representation of enzymatic hydrolysis of cellulose (after Erikssion, 1969)

Keeping the above facts in mind, the present work was undertaken to study the influence of the culture media, incubation period, temperature and hydrogen ion concentration (pH) on the cellulase (Cx) enzyme production of *Sclerotium rolfsii* Sacc.

Materials and Methods

Isolation of Pathogen

The pathogen used in this investigation was *Sclerotium rolfsii* Sacc.. which was isolated from infected foot region of *Solanum melongena* plant on potato-dextrose agar medium. The stock cultures were maintained in potato dextrose agar slants under refrigeration at 4ºC (Chaurasia, 2000; Chaurasia *et al.* 2014 b).

Chemicals

All chemicals was of analytical grade. Agar-agar was obtained from Merck Germany. Carboxymethyl cellulose (CMC) was obtained from Whatman Ltd. England. All other chemicals were obtained from sigma chemicals Co. Ltd. England.

Culture conditions and Enzyme production:

(a) Effect of culture media

To see the effect of different broth media on cellulase (Cx) enzyme production the following ten media of composition (g/L) were tested.

1. Asthana and Hawker's

Glucose 5, KNO_3 3.5, KH_2PO_4 1.75, $MgSO_4$. $7H_2O$ 0.75, Distilled Water to 1 L.

2. Basal mucor

Dextrose 10, Asparagine 2, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.25, Thiamine chloride
0.5, Distilled Water to 1 L.

3. Brown's

$MgSO_4.7H_2O$ 0.75, KH_2PO_4 1.25, Asparagine 2, Dextrose 20, Starch 10, Distilled
Water to 1 L.

4. Czapek's

$NaNO_3$ 2, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5, KCl 0.5, $FeSO_4.7H_2O$ 0.01, Sucrose 30,
Distilled Water to 1 L.

5. Dextrose-asparagine phosphate

Dextrose 30, $MgSO_4.7H_2O$ 0.5, Asparagine 1, KH_2PO_4 1.5, Distilled Water to 1 L.

6. Elliot's

Dextrose 5, Asparagine 1, Sodium Carbonate 1.06, $MgSO_4.7H_2O$ 0.5, KH_2PO_4
1.36, Distilled Water to 1 L.

7. Fernando's

$MgSO_4$ 5, KH_2PO_4 6.8, Asparagine 5, Glucose 15, Distilled Water to 1 L.

8. Glucose-dox

$MgSO_4.7H_2O$ 0.5, KH_2PO_4 1, $FeSO_4.7H_2O$ 0.01, $NaNO_3$ 2, KCl 0.5, Glucose 15,
Distilled Water to 1 L.

9. Glucose-nitrate
Glucose 10, $NaNO_3$ 1, KH_2PO_4 1, Distilled Water to 1 L.

10. Potato dextrose
Peeled potato slices 200, Dextrose 20, Distilled Water to 1 L.

The pathogen was grown in 150 ml Erlenmeyer flask containing 25 ml of various broth media. These flasks were sterilized at 15 lb/sq. in pressure for 15 min. The inoculum in each case consisted of a single disc of 8 mm diameter cut-out from the margin of a freshly grown colony of the pathogen on Potato-dextrose agar medium. The inoculated flasks were incubated for 9 days at 30ºC. After 9 days of incubation, the mycelium was removed culture filtrate was collected in separate flasks and the filtrates were then centrifuged at 10,000 rpm for 20 minutes at 4ºC using ultracentrifuse. After centrifugation, the clear supernatant liquids thus obtained were decanted and used as enzyme extract.

(b) Effect of incubation period
After selection of broth medium, the effect of different incubation periods, i.e., 3, 6, 9, 12, 15 and 18 days was tried to study their influence on the cellulase (Cx) enzyme production. For this study, the pathogen was grown in 150 ml Erlenmeyer flasks containing 25 ml of Potato-dextrose broth medium. Each flask was inoculated aseptically and then incubated at 30ºC. After 3, 6, 9, 12, 15 and 18 days of incubation, the culture filtrate was obtained and centrifuged at 10,000 rpm for 20 minutes. The clear supernatants were used as enzyme extract.

(c) Effect of temperature
The effect of different temperatures i.e., 15, 20, 25, 30, 35, 40 and 45ºC was tried to study their influence on the production of cellulase (Cx) enzyme by *Sclerotium rolfsii*. The pathogen was grown in 150 ml Erlenmeyer flask containing 25 ml of the Potato-dextrose broth medium. After inoculation, the inoculated flasks were incubated at 15, 20, 25, 30, 35, 40 and 45ºC temperatures for 9 days. After 9 days the culture filtrates were centrifuged to obtain supernatants which were used as enzyme extract.

(d) Effect of pH
To see the effect of different pH on cellulase (Cx) enzyme production, the pathogen was grown in Potato-dextrose broth medium with pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The pH of the medium was adjusted with 1N NaOH and 0.1 N HCl. After inculation with pathogen, the inoculated flasks were incubated at 30ºC for 9 days. At the end of the incubation period, the culture filtrate was obtained and centrifuged at 10,000 rpm for 20 minutes. The clear supernatants were used as enzyme extract.

Assay of cellulase (Cx) enzyme activity
Enzyme extracts obtained were assayed for the presence and activity of cellulase (Cx). The activity of cellulase (Cx) was measurd by using standard viscometric method (Chaurasia *et al.* 2013a; Chaurasia *et al.* 2013b; Chaurasia *et al.* 2013c; Chaurasia *et al.* 2014 a).
Oswald viscometers were clamped in stands which were fixed vertically in water bath, with temperature adjusted to 28ºC. For the assaying of cellulase (Cx) enzyme, the following freshly prepared substrate components were used :

1.2% carboxymethyl cellulose	**….**	**….**	**3.5 ml**
Distilled water	**….**	**….**	**1.5 ml**
Citrate phosphate buffer (pH 5.0)	**….**	**….**	**1.5 ml**

At the time of determination of cellulase (Cx) enzyme activity, desired substrate component was taken into the stalk bulb of viscometer. Then, 1.5 ml of freshly prepared enzyme extract was poured into viscometer and soon efflux time of the enzyme reaction mixture was determined at the intervals of 0, 20, 40, 60 and 80 minutes. Efflux time for 8.0 ml of distilled water was also noted in each viscometer.

Determination of per cent loss in viscosity
Per cent loss in viscosity was calculated with the help of the following formula (Chaurasia *et al.* 2013 a; Chaurasia *et al.* 2013 b; Chaurasia *et al.* 2013 c; Chaurasia *et al.* 2014 a).

$$\text{Percent loss in viscosity} = X\ 100\ \frac{ET_0 - ET_t}{ET_0 - ET_W}$$

Where,
ET_0 = Efflux time in seconds at zero time/control.
ET_t = Efflux time in seconds at any specific interval of time.
ET_w = Efflux time in seconds for distilled water.

Determination of relative enzyme activity (REA)
Values for per cent loss in viscosity were determined for 0, 20, 40, 60 and 80 minutes reaction time. These values were then plotted against the reaction time, thus a curve was obtained and from this curve the time to bring a 25 per cent loss in viscosity was determined. Relative enzyme activity (REA) was then calculated by the following formula:

$$REA = \frac{1}{\text{Time for 25\% loss in viscosity}} X\ 1000$$

Results and Discussion
Effect of culture media on the production of cellulase (Cx)
The effect of ten different broth media, viz., Asthana and Hawker's, Basal mucor, Brown's, Czapeks, Dextrose-asparagine phosphate, Elliot's, Fernando's, Glucose-dox, Glucose-nitrate and Potato-dextrose were tried to study their influence on the production of cellulase (Cx). It is clear from the results (Table 1 and Figure 2) that *Sclerotium rolfsii* produced good amount of cellulase in Potato-dextrose, Elliot's, Dextrose asparagines Phosphate, Brown's, Fernando's and Basal mucor media. Remaining of other's media i.e., Asthana and Hawkers, Czapek's, Glucose-dox and Glucose-nitrate, no cellulase was detected. Perhaps, nitrogen source and other ingradients of the medium may interfered with the cellulase production of pathogen. Rajmane and Koreker (2012) reported that nitrites and nitrates containing media are toxic for cellulase production of post harvest fungi. Several workers like Ritter (1909), Bach (1927), Thornton (1956), Sarbhoy (1963, 1977), Baijal (1967), Bilgrami (1975) and Chaurasia et.al. (2013) having also similar opinion as they have reported that nitrate containing media are very toxic to several members of microorganisms. In the present study the maximum production of cellulase was observed in Potato-dextrose medium, in which maximum relative enzyme activity (REA 124.06) has been calculated. Next to Potato-dextrose medium, Elliot's (REA 112.10) Dextrose-asparagine phosphate (REA 104.49) Brown's (REA 97.08 and Fernando's media (REA 95.23) have been found to be

satisfactory in which appereciable amount of cellulase was determined. Comparatively, in Basal mucor medium (REA 54.05) poor cellulase production was recorded. Since none of the media used in the present work were supplemented with cellulosic substrates it would appear that cellulase production of *Sclerotium rolfsii* is constitutive in nature.

Table 1: Effect of different culture media on the production of cellulase (Cx)

Media	Enzyme activity (% Loss in viscosity) Reaction time (in mts.)				REA
	20	40	60	80	
Asthana and Hawker's	0.0	0.0	0.0	0.0	0.00
Basal mucor	27.0	34.2	41,0	48.5	54.05
Brown's	48.5	67.5	73.1	79.0	97.08
Czapek's	0.0	0.0	0.0	0.0	0.00
Dextrose-asparagine phosphate	52.2	68.8	78.0	83.7	104.49
Elliot's	56.0	75.2	84.0	86.4	112.10
Fernando's	47.6	68.8	86.0	88.8	95.23
Glucose-dox	0.0	0.0	0.0	0.0	0.00
Glucose-nitrate	0.0	0.0	0.0	0.0	0.00
Potato-dextrose	62.0	81.6	86.0	91.5	124.06

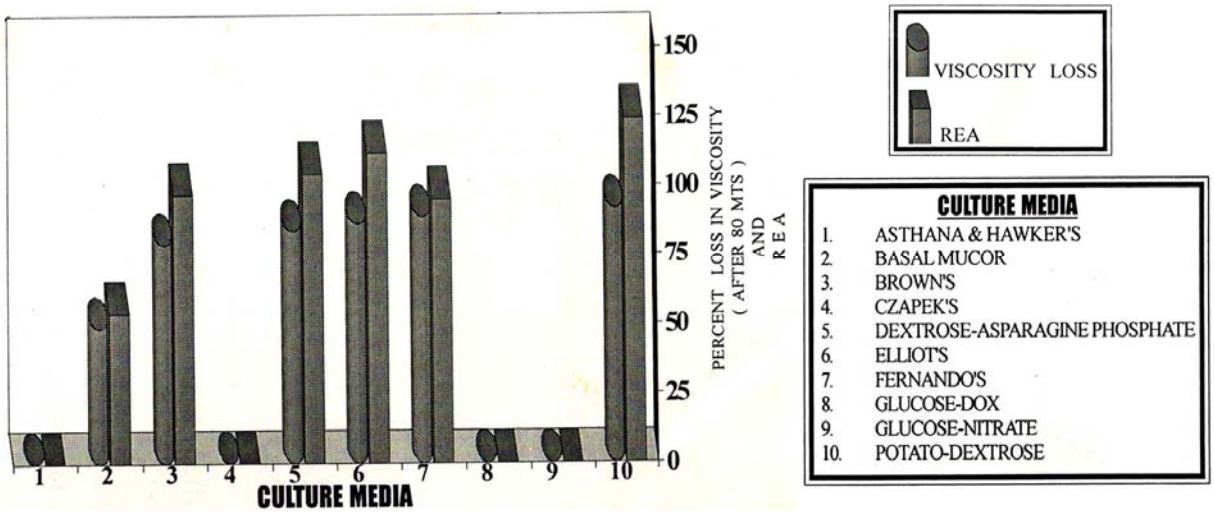

Fig 2: Effect of different culture media on cellulase (Cx) production by *Sclerotium rolfsii*

On the whole, it is concluded that Potato-dextrose medium was found to be the best for the maximum production of cellulase. Asthana and Hawker's, Czapeks, Glucose-dox and Glucose-nitrate media showed toxic effect for the production of cellulase in which no trace of cellulase enzyme was detected.

Effect of incubation period on the production of cellulase (Cx)

The incubation period is directly related with the production of cellulase enzyme and other metabolic activity up to certain extent. *Sclerotium rolfsii* showed most active cellulase production along with different incubation period. In this study, *Sclerotium rolfsii* was cultured at 30°C on Potato-dextrose broth medium for 3, 6, 9, 12, 15 and 18 days incubation period. In the present study the result were recorded dipicted in Table 2 and Figure 3. The results revealed that *Sclerotium rolfsii* could be able to synthesize cellulase enzyme with in a short time i.e., within 3 days. The cellulase enzyme activity reapidly increased with the length of incubation period upto 9 days. In 9 days of incubation period, the maximum relative enszyme activity (REA 120.06) was recorded. Further increase in length of incubation upto 18 days, had no effect on cellulase

production as seen by the gradual reduction in the relative enzyme activity. It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Nochure *et al.* 1993).

Table 2: Effect of different length of incubation period on the production of cellulase (Cx)

Incubation period	Enzyme activity (% Loss in viscosity) Reaction time (in mts.)				REA
	20	40	60	80	
3 Days	36.5	62.0	74.5	80.0	73.04
6 Days	55.0	77.3	81.0	84.6	110.01
9 Days	62.0	81.6	86.0	91.5	124.06
12 Days	52.3	70.0	78.0	83.5	104.60
15 Days	46.4	63.5	73.0	78.6	92.85
18 Days	42.0	58.2	70.5	75.5	84.03

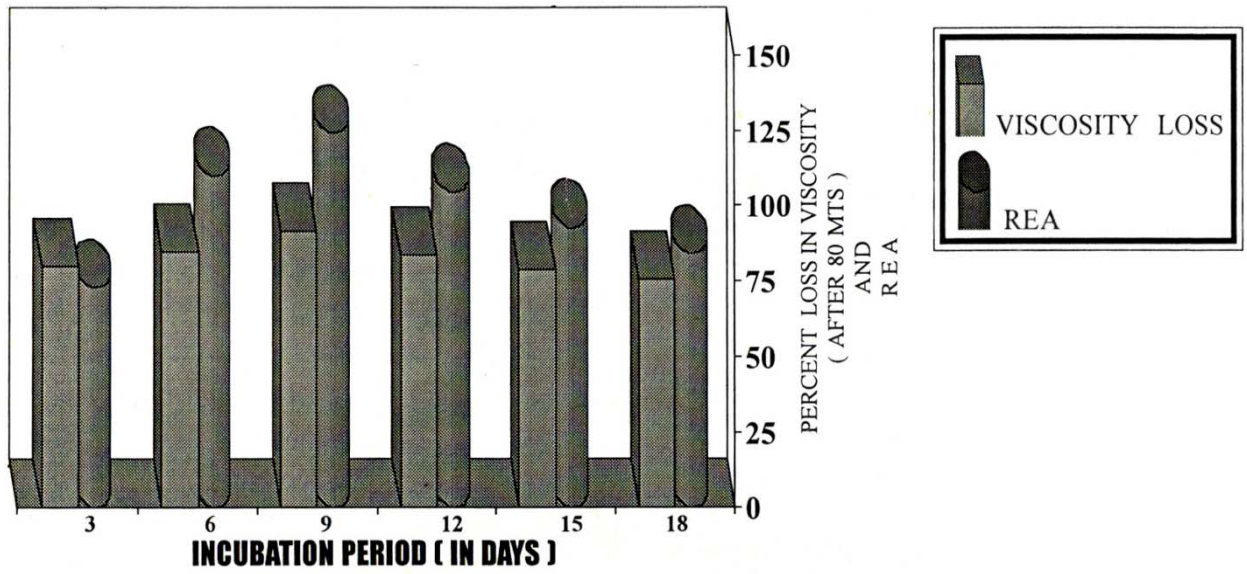

Fig 3: Effect of incubation period on cellulase (Cx) production by *Sclerotium rolfsii*.

On the basis of for said findings, it is concluded that *Sclerotium rolfsii* has capability of producing cellulase enzymes as they were detected in 3 days of incubation period. 9 days length of incubation was found to be the best for the maximum production of cellulase enzyme. Similar results have also been recorded by Tarek (2013) with sugarbeet pathogen *Sclerotium rolfsii*. Bateman (1969) reported that *Sclerotium rolfsii* produced maximum cellulase enzyme between 6 to 10 days of incubation period.

Effect of temperature on the production of cellulase (Cx): Temperature is one of the most important factors that influence the secretion of fungal cellulase enzyme. In order to study the effect of different temperatures, *Sclerotium rolfsii* was cultured on Potato-dextrose broth medium at different temperatures, viz., 15, 20, 25, 30, 35, 40 and 45°C for 9 days. It is evident from the results Table 3 and Figure 4 that the cellulase production was found to less at very low temperature i.e. at 15°C. At 15°C temperature, 60.5% loss in viscosity of substrate was recorded at the end of reaction time (REA 64.43). As the temperature increased, the production of cellulase enzyme was increased and maximum cellulase production recorded in culture filtrate of 30°C. Further increase of temperature upto 35°C, the cellulase production was considerably reduced. In the culture filtrate of 40 and 45°C temperature, no trace of cellulase enzyme production was recorded. These results are in agreement with results reported by Hussain et.al. (2012). Who reported an optimal temperature of cellulase activity in the range of 30-35°C for soft rot filamentous fungi *Paecilomyces variotii*. Many workers have reported that 30°C was the optimum temperature for cellulase production by several fungal genera and species including *Fusarium oxysporum* (El-Said *et al.* 2006), *Chaetomium globosum* (El-Said and Saleem, 2008), *Aspergillus Niger* (Ja' afaru and Fagade, 2010 and Ilyas *et al.* 2011), *Alternaria citri* and *Cochliobolus spicifer* (El-said *et al.* 2014). However, 30-35°C was the optimum for cellulase produced by *Chaetomium globosum* (El-Said, 2001) and

Trichoderma harzianum (Ahmed *et al.* 2009). Results of other workers stated that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Murao *et al.* 1988; Lu *et al.* 2003).

Table 3: Effect of different temperatures on the production of cellulase (Cx)

Temperature (^{0}C)	Enzyme activity (% Loss in viscosity) Reaction time (in mts.)				REA
	20	40	60	80	
15	32.2	50.5	56.0	60.5	64.43
20	48.0	67.8	75.5	79.5	96.06
25	54.0	73.0	80.5	85.3	108.10
30	62.0	81.6	86.0	91.5	124.06
35	42.3	57.1	62.2	65.1	84.60
40	0.0	0.0	0.0	0.0	0.00
45	0.0	0.0	0.0	0.0	0.00

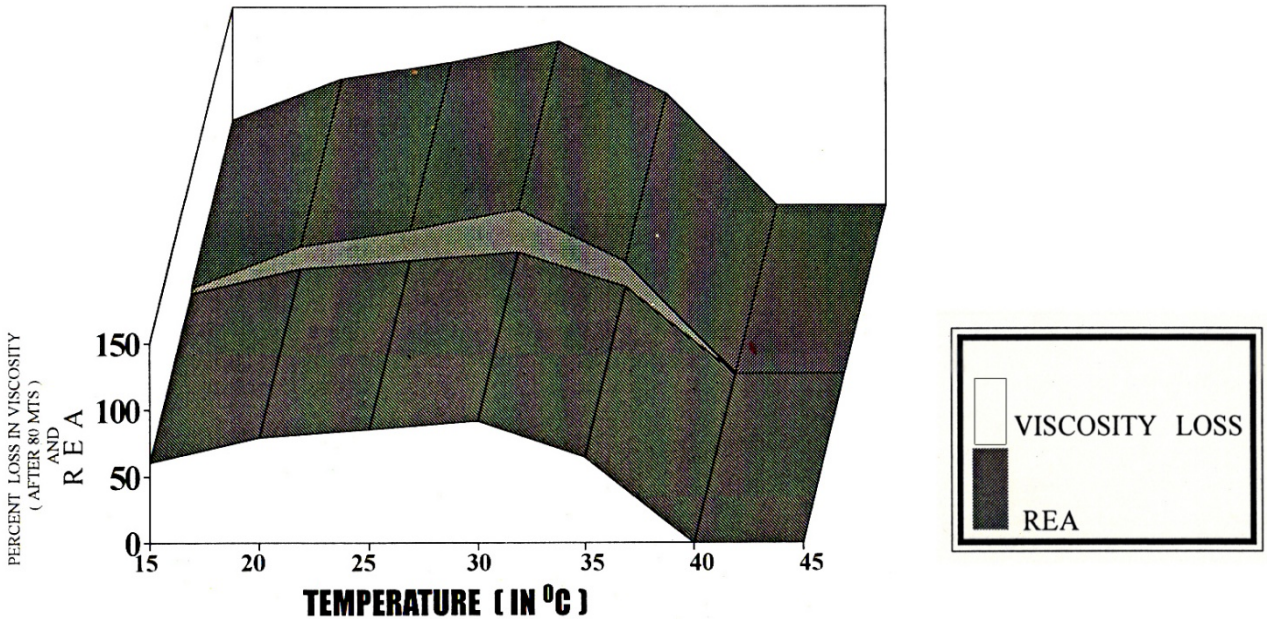

Fig 4: Effect of temperature on cellulase (Cx) production by *Sclerotium rolfsii*.

In the light of above findings, it is concluded that Sclerotium rolfsii was able to produce cellulase enzyme at various temperatures. The 30°C temperature was found to be optimum and most suitable for the production of cellulase enzyme.

Effect of hydrogen ion concentration (pH) on the production of cellulase (Cx):

The hydrogen ion concentration (pH) of the medium is greatly affected the production of cellulase enzyme. The effect of different pH viz., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were tried on the production of cellulase enzyme. The pathogen *Sclerotium rolfsii* was cultured on Potato-dextrose broth having, different pH values, at 30°C temperature for 9 days. After 9 days incubation period, in culture filtrate of each set of experiment, the enzyme production was determined as per cent loss in viscosity of substrate and relative enzyme activity (REA) was also calculated. The results obtained during the investigation was tabulated in Table 4 and represented graphically in Figure 5. From the results, it is evident that *Sclerotium rolfsii* possesses great capacity to synthesize cellulase enzyme at a wide range of pH , i.e., pH 3.0 to 9.0. At low pH value i.e., pH 3.0, about 72.3% loss in viscosity of substrate was recorded (REA 86.20) which increased with increase in pH value upto pH 5.0, at which 91.5% loss in viscosity (REA 124.06) have been recorded. On further higher pH values, i.e., from pH 5.0 to 9.0, the cellulase production gradually decreased. The pH 9.0 was found to be the most unfavourable for the production of cellulase, as in the culture filtrate of this pH value comparatively poor cellulase enzyme activity was determined. It may be due to the induction of cellulase occurs at the stage of high metabolic activity when the pH of the medium lies in acidic range of pH. The pH 5.0 has already reported optimum for cellulase production (Richhariya, 1984), Because very less enzyme is required to produce sugar at this pH.

Table 4: Effect of different pH on the production of cellulase (Cx)

pH	Enzyme activity (% Loss in viscosity) Reaction time (in mts.)				REA
	20	40	60	80	
3.0	43.1	60.2	67.0	72.3	86.20
4.0	50.2	68.5	78.3	83.5	100.40
5.0	62.0	81.6	86.0	91.5	124.06
6.0	55.2	72.5	82.1	87.2	110.49
7.0	51.0	65.6	71.4	75.5	102.04
8.0	45.0	56.3	63.2	66.4	90.00
9.0	41.3	49.4	55.2	58.6	82.64

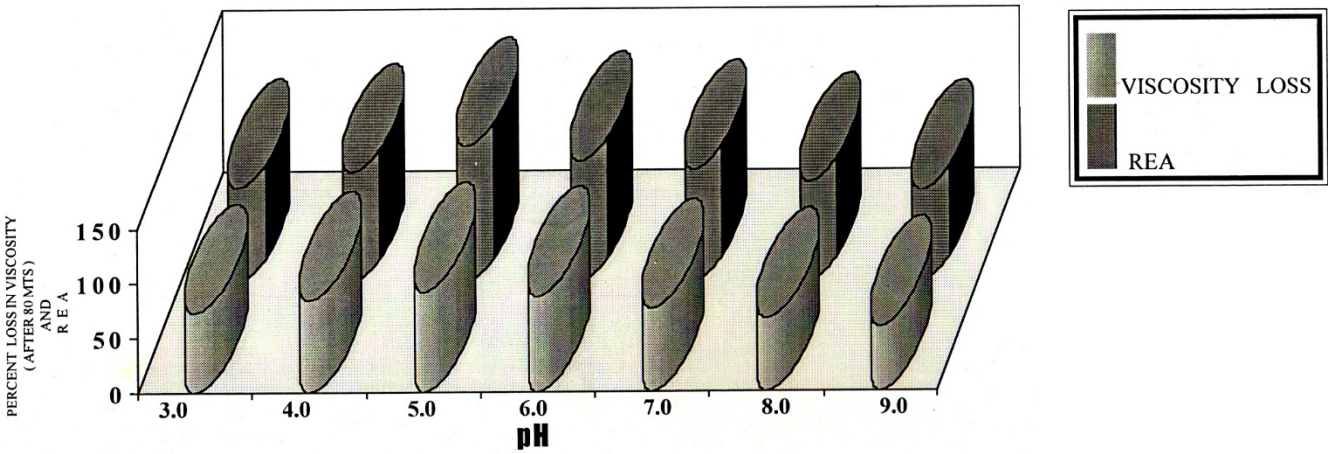

Fig 5: Effect of pH on cellulase (Cx) production by *Sclerotium rolfsii*.

In the light of above findings, it is concluded that *Sclerotium rolfsii* possesses capability to synthesize cellulase enzyme at a wide range of pH, i.e., from pH 3.0 to 9.0. pH 5.0 was found to be most suitable and favourable for the maximum production of cellulase. Richhariya (1984) has also found the maximum production of cellulase in the culture filtrate of that medium which was adjusted at pH 5.0, in case of tomato isolate of *Sclerotium rolfsii*. Similar results have also been recorded by Hussain et.al. (2012) in case of *pacilomyces variotii*. Baig (2005), observed that cellulase production of *Trichoderma lignorum* reached the maximum value at pH 5.6 – 5.8. Ahmed et.al. (2009) and Gautam et.al. (2010) reported that, the most suitable pH for cellulases production by *Trichoderma harziarum* and *Trichoderma viride* was 5.5

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