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Research Article

Production of Amylase Enzyme by *SCLEROTIUM ROLFSII* SACC. under Different Cultivation Conditions

***[1]amit Kumar Chaurasia, [1]Shridha Chaurasia,**
[2]Shubha Chaurasia, And [3]Sushmita Chaurasia
[1]Department of Botany, Govt. P.G. College, Tikamgarh (M.P.) 472001 India.
[2]Department of Boatany, Govt. College Palera District Tikamgarh (M.P.) 472221 India.
[3]Department of Botany, Govt. College Jatara District Tikamgarh (M.P.) 472118 India.
Corresponding Author e-mail:- dr.amitkumarchaurasia@gmail.com

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ABSTRACT:
Amylase production and its optimaization has been studied in *Sclerotium rolfsii*. Various culture media, wide range of temperature and pH were tested to obtain maximum amylase production. The activity of amylase was determined by cup-plate method. Out of ten media, only six media i.e., Basal mucor, Brown's, Dextrose-asparagine phosphate, Elliot's, Fernando's, and potato-dextrose media were proved to be producer of amylase. No trace of amylase enzyme has been detected in the culture filtrates of remaining four media i.e., Asthana and Hawker's, Czapek's, Glucose-dox and Glurcase'Nitrate media. Amongst six amylase producer media, Elliot's medium was found to be the best for the maximum production of amylase. Nine days of incubation period was also found to be the best for maximum production of amylase enzyme. The *Sclerotium rolfsii* was able to produce amylase enzyme at wide range of temperatures i.e., from 15ºC to 35ºC. the maximum production of amylase has been detected in culture filtrates of those flasks which were incubated at 30ºC. Therefore, 30ºC temperature was found to be the best suitable for the maximum production of amylase enzyme. At extreme acedic pH (i.e., pH 3.0) and extreme alkaline pH (i.e., pH 9.0) have no remarkable effect on the production of amylase. Slightly acedic pH (i.e., pH 5.0 and 6.0) found to be favorable for amylase production in significant quantity. pH 6.0 was found to be optimum and most suitable for the maximum production of amylase enzyme.

Keywords: Sclerotium rolfsii, Amylase, Culture media, Temperature, pH.

INTRODUCTION

In the last few decades micro organisms have made a useful contribution to the industrial enzyme production, including amylases [4,26,21,5,7,29,36]. Amylase is an important enzyme for its application in food, baking, brewing, paper, drink, textiles, detergents, pharmaceutical and sugar industry. It is also extensively used commercially for the production of sugar syrups from starch which consist of glucose, maltose and higher oligosaccharides. [4,24]. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expended into many other fields such as clinical, medical and analytical chemistry [26].

Enzymatic hydrolysis has proven its superiority over acid hydrolyses as there is no need for high temperature or pressure and very low pH. So there are many amylase producing microorganisms which have been tested for being

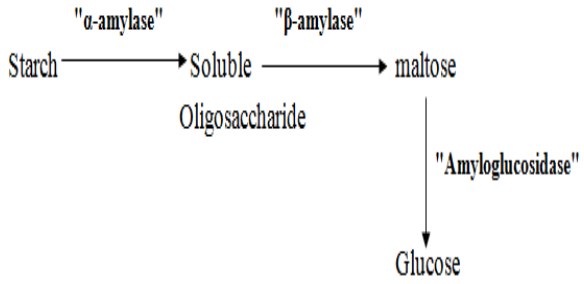
utilized in industry for starch hydrolysis by enzymatic methods and have proven to be highly economic [8,20,39]. Microbial conversion of starch into useful byproducts is a complex process involving combined action of following three enzymes:

(i) α-amylase (Alpha-amylase) – Reduces the viscosity of starch by breaking down the bonds at random, therefore producing varied sized chains of glucose.

(ii) β-amylase (Beta-amylase) – Breaks the glucose-glucose bonds down by removing two glucose units at a time, thereby producing maltose.

(iii) Amyloglucosidase (AMG) – Breaks successive bonds from the non-reducing end of the straight chain, producing glucose.

Degradation of starch takes place as follows by the action of these three enzymes:



In this way, the enzyme amylase helps in the degradation of starch 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.

Most of the non-pathogenic fungi have been reported to produce the amylase enzyme, during their growth and metabolic activity [22,34,18,19,2,24,4,26,1,21,7,29,5,35] very little is known about the amylase producing phytopathogenic fungi [11,13,14,30]. In view of the above and also due to the lack of any information regarding the amylase producing capacity of *Solanum melongena* foot rot pathogen, *Sclerotium rolfsii* Sacc. work on its enzymological aspects was undertaken in which the effects of various culture media, temperature and hydrogen ion concentration on the production of amylase enzyme were studied by cup-plate assay method.

MATERIALS AND METHODS

Isolation of Microganism :

Sclerotium rolfsii sacc. was isolated from the foot region of infected brinjal (*Solanum melongena* Linn.) plant (Chaurasia et.al., 2013; Chaurasia et.al., 2014). The pathogen was maintained on potato dextrose agar medium slants under refrigeration at 4ºC. this was identified on the basis of morphological characteristics.

Effect of culture media:

To find out the suitability of amylase production, ten different culture media of composition (g/L) shown below 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 to1 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.

For the production of amylase enzyme, the pathogen *Sclerotium rolfsii* was grown in 150 ml Erlenmayer flasks containing 25 ml of the respective broth medium. These flasks were sterilized at 15 lb/sq in pressure for 15 minutes. After sterilization each flask was inoculated by a 8.0 mm disc taken from the periphery of 72 hours old colony of the test pathogen growing on potato dextrose agar medium. The flasks were incubated at 30ºC for 3, 6, 9, 12, 15 and 18 days. Three replicates were taken in each case. After each incubation period, the culture filtrate of each set was collected and used for enzyme extraction.

Effect of temperature:

After selection of culture medium, the effect of seven different temperatures, i.e. 15, 20, 25, 30, 35, 40 and 45ºC was tried to study their influence on the production of amylase enzyme. The *Sclerotium rolfsii* was cultured, in 150 ml Erlenmeyer flask containing 25 ml of Elliot's broth medium (pH 6.0) at different above said temperatures for 9 days. This medium was found to be a suitable one for the maximum production of amylase enzyme. After 9 days of incubation, the culture filtrate of each set was used for enzyme extraction. Each set was run in triplicates.

Effect of pH:

To study the effect of different pH, the selected medium i.e., Elliot's broth medium was taken and different pH values, viz., pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were adjusted by addition of 0.1 N NaOH or 0.1N HCl. 25 ml of Elliot's broth medium, adjusted at desired pH was inoculated and incubated at 30ºC for 9 days. After incubation, the culture filtrate of each set was used for enzyme extraction. Each set was run in triplicates.

Enzyme extraction :

After desired incubation, fungal mat was removed from the medium and the culture filtrates of pathogen were collected in separate flasks by filtration under suction. The culture filtrates thus obtained were centrifuged at 10,000 rpm at 4ºC for 20 minutes. After centrifugation, the clear supernatant liquids obtained decanted and used as the crude enzyme preparations.

Assay of enzyme activity:

Enzyme preparations thus obtained were assayed for the activity of amylase. The activity of amylase was determined by cup plate assay method [19,13,14].

Starch agar medium of the following composition was used for cup plate assay method.

Soluble starch	.....	.....	**10.00 g.**
Na_2HPO_4	.....	.....	**2.84 g.**
NaCl	.....	.....	**0.35 g.**
Agar agar	.....	.....	**20.00 g.**
Distilled water	.....	.....	**1000 ml.**

Twenty five ml of melted starch agar medium was poured into 90 mm diameter sterilized Petridishes and allowed to solidify at room temperature. Then a cavity was made in the centre of each Petridish with the help of cork borer (10 mm diameter). After this, the bottom of the cavity of each Petridish was sealed by adding two drops of melted agar. 0.2 ml of enzyme extract (culture filtrate) of pathogen was added to the cavity with the help of micropipette and then incubated at 30°C temperature. After 24 hours, the petridishes were treated with Logol's iodine solution (Iodine, 5.0 g; Potassium iodide 10.0 g; Distilled water, 1000 ml). After iodine treatment a clear non-blue zone appeared around the cavity. The diameter of non-blue zone was measured in mm and the activity of amylase expressed after substracting the diameter of cavity from the

diameter of non-blue zone. Each test runs in triplicate.

RESULTS AND DISCUSSION

Effect of different culture media on the production of amylase:

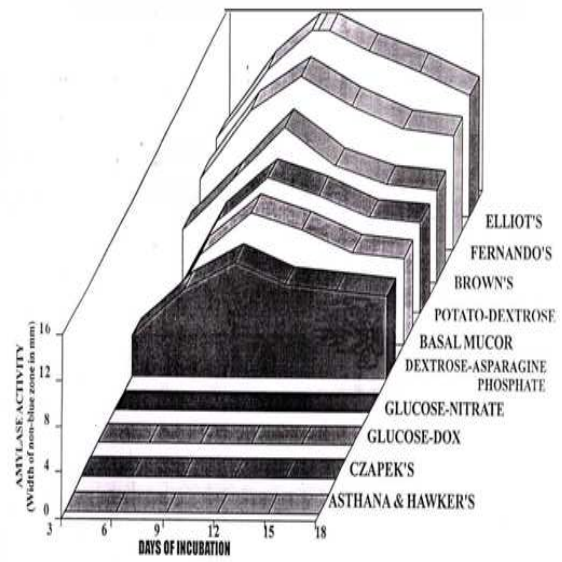
The effect of ten different culture media viz., Asthana and Hawker's, Basal mucor, Brown's Czapek's, Dextrose-asparagine phosphate, Elliot's, Fernando's, Glucose-dox, Glucose-nitrate and Potato-dextrose were tried to study their influence on the production of amylase. In each case, the production of amylase was observed after the incubation period of 3, 6, 9, 12, 15 and 18 days. The amylase producing capacity of *Sclerotium rolfsii* in different culture media is presented in Table 1, Fig. 1 and Plate 1.

Table 1 : Effect of different culture media on the production of amylase enzyme of *Sclerotium rolfsii*.

Media	Amylase activity (Width of non-blue zone in mm)*					
	Days of incubation					
	3	6	9	12	15	18
Asthana and Hawker's	0.0	0.0	0.0	0.0	0.0	0.0
Basal mucor	0.0	8.2	12.0	10.5	8.5	8.0
Brown's	4.5	9.0	13.5	10.2	8.7	8.2
Czapek's	0.0	0.0	0.0	0.0	0.0	0.0
Dextrose-asparagine phosphate	3.5	8.0	10.5	8.5	8.0	7.5
Elliot's	6.5	12.5	16.5	14.2	12.5	10.5
Fernando's	6.0	12.0	15.0	12.5	10.5	10.0
Glucose-dox	0.0	0.0	0.0	0.0	0.0	0.0
Glucose-nitrate	0.0	0.0	0.0	0.0	0.0	0.0
Potato-dextrose	0.0	8.3	12.1	10.6	8.7	8.3

* After deducting the cavity of 10.0 mm diameter.

Fig. 1. : Effect of different culture media on the production of amylase enzyme of *Sclerotium rolfsii*.



From the results, it is clear that *Sclerotium rolfsii* was found to be capable of producing amylase enzyme in the Basal mucor, Brown's, Dextrose-asparagine phosphate, Elliot's, Fernando's and Potato dextrose culture media. No trace of amylase enzyme has been detected in the culture filtrate of Asthana and Hawker's, Czapek's, Glucose-dox and Glucose-nitrate media and thus these four media have not favoured the production of amylase enzyme. Chaurasia (2013) has reported that Asthana and Hawker's, Czapek's, Glucose-dox and Glucose-nitrate broth media did not support the growth as in all these four media *sclerotium rolfsii* was unable to grow. Perhaps nitrogen source and other ingradients of the medium may interfered with the growth of pathogen and production of amylase enzyme. Several workers like Ritter [31], Thornton [38], Sarbhoy [32,33] and Bilgrami [10] having also similar opinion as they have reported that nitrate containing media are very toxic to several members of microorganisms. Chaurasia [12] reported the inhibitory effect of inorganic nitrogen sources on the growth of *Phytophthora parasitica var. Piperina* and a decrease in the rate of oxygen uptake.

Amongst the six amylase producer media, Elliot's medium was found to be the best for the maximum production of amylase and Fernando's medium found to be the next. Besides these two media, Basal mucor, Brown's, and Potato dextrose media have been found to be satisfactory and Dextrose-asparagine phosphate was found to be poor for the production of amylase enzyme.

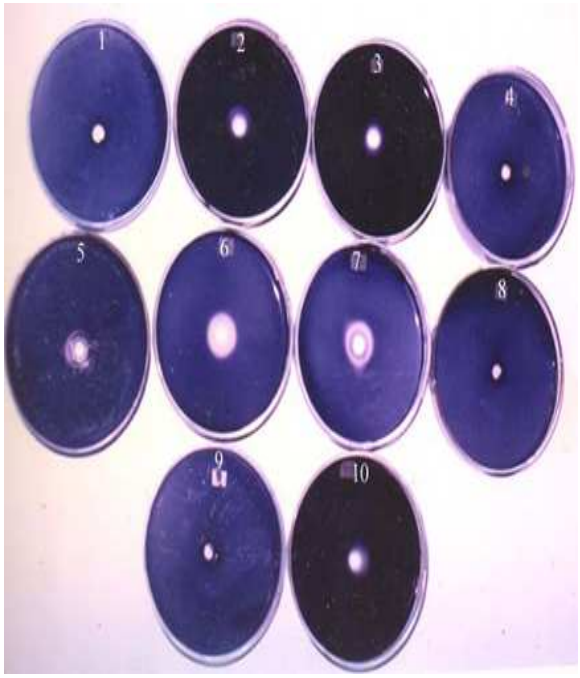


Plate 1. Showing the effect of following culture media on the production of amylase (After 9 days of inoculation period).

1. Asthana and Hawker's medium.

2. Basal mucor medium.

3. Brown's medium.

4. Czapeks medium.

5. Dextrose-asparagine phosphate medium.

6. Elliot's medium.

7. Fernando's medium.

8. Glucose-dox medium.

9. Glucose-nitrate medium.

10. Potato-dextrose medium.

From the results, it was also observed that amylase production was gradually increased with the increase in the incubation period upto 9 days. After 9 days of incubation, further increase in incubation periods has no effect, but rather resulted in reduction of amylase activity. Similar results have also been reported in various fungi by several workers, e.g., *Cephalosporium* species (Mangallam et.al., [23]), *Laphotrichus ampullus* (Pathak and Agrawal, [28]), *Myrothecium verrucaria*, *Fusarium equiseti*, *Sporotrichum xylophila* (Pandey and Saxena, [27]). It has been also reported that amylase is produce in the primary phase of growth and thereafter the activity decline either due to decrease in production or due to enzyme degradation [5]. The decrease enzyme activity due to end product repression by polymethylgalacturonase (PMG) and cellulase (Cx) with reaches a maximum value in 9 days incubation can not be ruled out [14].

From the above results it can be concluded that Elliot's medium was found to be the best for the production of amylase. Fernando's medium was recorded as next and Dextrose asparagine-phosphate medium proved to be poor for amylase production. The nine days incubation period was recorded optimum for the maximum production of amylase enzyme. It is also concluded from the experimental results that the type of culture medium and the duration of the incubation period gradually influenced the production of amylase enzyme. The enzyme are constitutive is clearly shown by their production in good amounts in the medium lacking starch materials.

Effect of different temperatures on the production of amylase :

To investigate the effect of different temperatures viz., 15, 20, 25, 30, 35, 40 and 45°C, the *Sclerotium rolfsii* was cultured on Elliot's medium for 9 days. Inoculated flasks were kept at above said different temperatures for 9 days and than culture filtrate was taken for assaying the activity of amylase enzyme.

In the present study, the results were recorded dipicted in Table 2 and represented graphically in Fig. 2 and Photographically in Plate 2.

Table 2: Effect of different temperatures on the production of amylase enzyme of *Sclerotium rolfsii* in Elliot's medium.

Temperature (°C)	Amylase activity (Width of non-blue zone in mm)*
15	12.1
20	14.5
25	16.0
30	16.5
35	12.4
40	0.00
45	0.00

*After deducting the cavity of 10.0 mm diameter.

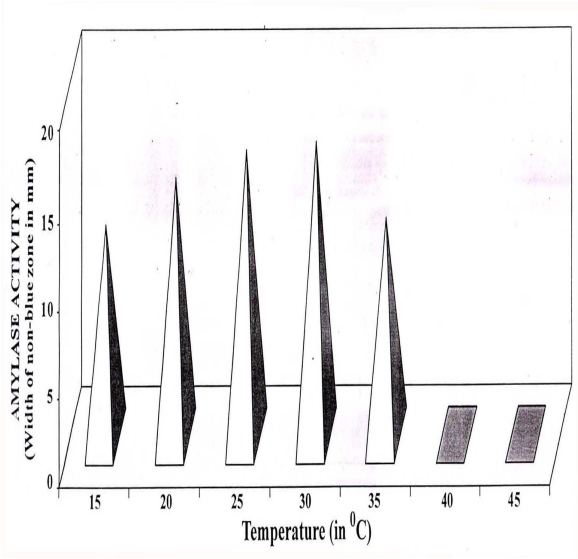


Fig. 2. : Effect of different temperatures on the production of amylase enzyme of *Sclerotium rolfsii* in Elliot's medium.

It is evident from the Table 2, Fig. 2 and Plate 2, that *Sclerotium rolfsii* was able to synthesize amylase enzyme between wide range of temperature, i.e., from 15 to 35°C. The maximum production of amylase enzyme was detected in the culture filtrates of those flasks which were kept at 30°C. Therefore, 30°C was found to be optimum temperature for the production of amylase. 25°C temperature was also proved to be equally good as 30°C. Above 30°C, at slightly higher temperature i.e. at 35°C, the amylase production was remarkably decreased. In culture filtrates of those sets, kept at 40 and 45°C, no trace of amylase enzyme was detected. Recently Chaurasia et.al. [15] has reported that higher temperature i.e., 40 and 45°C were found to be unfavorable for the growth of *Sclerotium rolfsii* and in these temperature the pathogen was unable to grow. This may be due to the detrimental or lethal effect of pathogen. These findings clearly indicate the important role of temperature in the production of amylase enzyme.

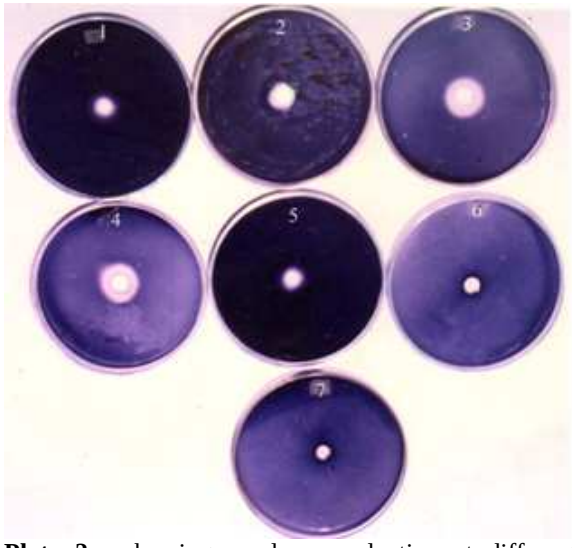


Plate 2. showing amylase production at different temperatures (After 9 days of inoculation period).

1. **15°C**
2. **20°C**
3. **25°C**
4. **30°C**
5. **35°C**
6. **40°C**
7. **45°C**

from the above result, it is concluded that 30°C temperature was found to be the best for the maximum production of amylase enzyme. These results are corelated with the findings of Mukharjee and Majumdar [25], Alva et.al [5], Bhattacharya et.al. [9] and Dalal et.al. [17] who have reported the 30°C is optimum for production of amylase. Incidentally results regarding the influence of temperature on the amylase

production showed a great deal of corelation with temperature effect on fungal growth. for both the fungal growth and amylase production the optimum temperature was 30°C.

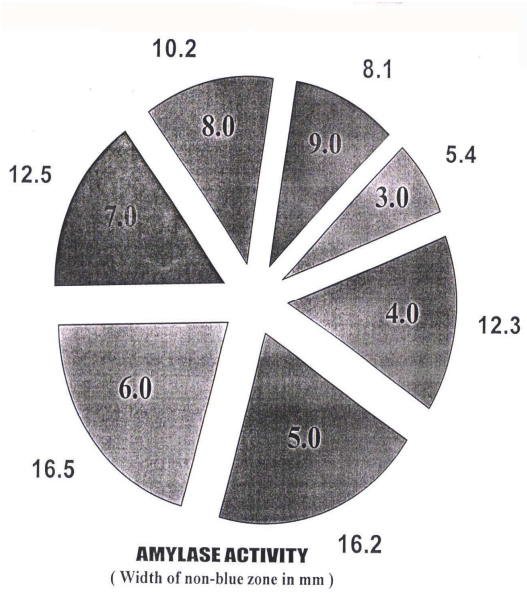
Effect of different hydrogen ion concentrations on the production of amylase

To study the effect of different hydrogen ion concentration (pH), the pathogen was culture on Elliot's medium having different pH values, viz., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 incubated at 30°C for 9 days. The results are presented in Table 3, Fig. 3 and Plate 3.

Table 3: Effect of different pH on the production of amylase enzyme of *Sclerotium rolfsii* in Elliot's medium.

pH	Amylase activity (Width of non-blue zone in mm)*
3.0	5.4
4.0	12.3
5.0	16.2
6.0	16.5
7.0	12.5
8.0	10.2
9.0	8.1

Fig. 3. : Effect of different pH on the production of amylase enzyme of *Sclerotium rolfsii* in Elliot's medium.



Results obtained during the present study clearly revealed that *Sclerotium rolfsii* was able to produce amylase on a wide range of pH i.e., from 3.0 to 9.0 pH. At extreme low pH i.e., 3.0 pH, the amylase production was very poor (non-blue zone 5.4 mm). The amylase production was gradually increased with the increase in pH values upto pH 6.0, which was found to be the best for amylase production. Further increase in pH values having no effect and production of amylase was gradually decreased. From the results it is also clear that slightly acidic pH (i.e., pH 5.0 to 6.0) favored the amylase production but in extreme acidic pH (i.e. pH 3.0) found to be unfavorable for the production of amylase. Extreme alkaline pH (i.e. pH 9.0) have not show any remarkable effect on the production of amylase in comparison to extreme acidic pH (i.e. pH 3.0). The effect of pH on the enzyme production indicate that the amylase is active in the pH range 3.0 to 9.0. This suggest that the enzyme would be useful in process that require wide range of pH change from highly acidic to slightly alkaline range and vice-versa. Similar result have also been recorded by Alva et.al. [5].

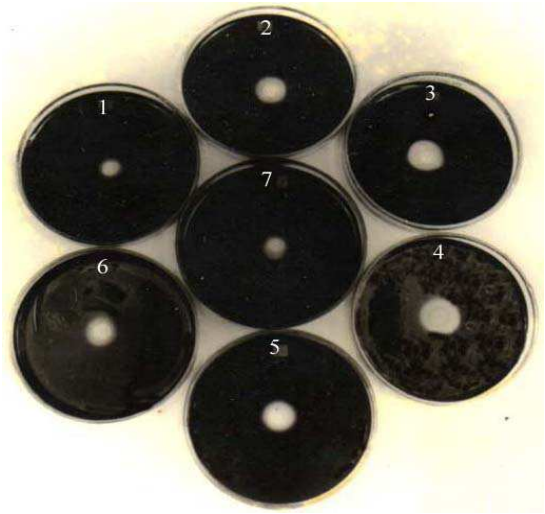


Plate 3. Showing amylase production at different pH (After 9 days of inoculation period).

1. **pH 3.0**
2. **pH 4.0**
3. **pH 5.0**
4. **pH 6.0**
5. **pH 7.0**
6. **pH 8.0**
7. **pH 9.0**

From the above result it is concluded that pH value 6.0 was found to be optimum for the maximum production of amylase enzyme.

CONCLUSION:

The production of an amylase enzyme is very sensitive to culture medium, incubation period, temperature and hydrogen ion concentration (pH). Therefore, the selection of suitable medium, optimum incubation period, optimum temperature and optimum pH are essential for the production of the amylase enzyme. In this study the effect of different culture media, length of incubation period, temperature and pH were studied. Out of ten culture media, Elliot's medium was found to be the best for the maximum production of amylase. It is also concluded that 9 day incubation period was found to be optimum for maximum production of amylase enzyme. Production of amylase enzyme was the best at 30°C. Further increase in temperature resulted in decrease of amylase production by the pathogen. Slightly acidic pH (i.e. pH 5.0 and pH 6.0) found to be favorable for amylase production in significant quantity. But pH 6.0 was found to be optimum for most suitable for the maximum production of amylase enzyme. This property makes the enzyme suitable for industrial production of paper, detergents, food, baking, brewing and textile.

Further studies were in progress in the purification and application of amylase in different commercial fields. The purified amylase can be used for various purposes in paper industries, detergent industries, food industries, baking industries, brewing industries, textile industries and pharmaceutical industries.

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