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RESEARCH ARTICLE

EFFECT OF CULTURE CONDITIONS ON THE PRODUCTION OF AMYLASE ENZYME BY *RHIZOPUS ORYZAE* (Went & Prins Geerl.).

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Abstract

Amylases are among the most important enzymes and have great significance in present-day biotechnology. Fungal amylase has large application in food and pharmaceutical industries. Considering these facts, the effect of different culture media, temperature and pH on amylase production by *Rhizopus oryzae* were studied. The activity of amylase was determined by cup plate assay method as described by Chaurasia et al. (2015).

In order to select the best suitable medium, the production of amylase enzyme was determined by growing the *Rhizopus oryzae* in eight different culture media for 3, 6, 9, 12, 15 and 18 days at 30^0C temperature. Among eight culture media, Brown's, dextrose-asparagine-phosphate, Glucose-dox and Glucose-nitrate media have not favoured the production of amylase as in the culture filtrate of these four media, no trace of amylase enzyme have been detected. While remaining four culture media (i.e. Molisch's, Elliots, Fernando's and Waksman's), *Rhizopus oryzae* was able to produce amylase enzyme. Amongst these four culture media, Fernando's medium was found to be the best for the production of amylase enzyme. Nine days of incubation period was also found to be the best for maxium production of amylase enzyme. For further study, Fernando's medium and nine days of incubation period were selected for amylase production.

To study the influence of temperature, *Rhizopus oryzae* was cultured on Fernando's medium (pH 5.0) for nine days at different temperatures viz., 15, 20, 25, 30, 35, 40, 45 and 50^0C. The *Rhizopus oryzae* was able to produce the amylase enzyme at wide range of temperature, ranging from 15^0 to 40^0C. The 30^0C temperature was found to be optimum for the maxiumum production of amylase enzyme. Above 40^0C, no trace of amylase production was recorded.

To study the effect of pH on amylase production, *Rhizopus oryzae* was cultured on Fernando's medium having different pH values, viz, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 at 30^0C for 9 days incubation period. The isolated pathogen *Rhizopus oryzae* was able to synthesized amylase enzyme at a wide range of pH, i.e., from pH 3.0 to 8.0. Extremely acidic pH (i.e. at pH 3.0), the activity of amylase was found to be less which increased gradually with increase in pH up to pH 5.0. Further

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increasing the pH, has no effect on the production of amylase enzyme. The pH 5.0 was found to be optimum for the maximums production of amylase enzyme.

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Introduction:-

Starch a homo-polysaccharideisan important and abundant food reserve and energy source in plant. It is found in seeds, fruits, leaves, bulbs and tubers (Bozic et al., 2011; Pathania et al., 2017). Structurally starch is a composite polymer of linear amylose (α-1, 4 liked D-glucose residues) and branched amylopectin (containing both α-1, 4 and α-1, 6 linked D-glucose residues) which degraded predominantly by hydrolytic enzymes called amylase enzymes.

Amylase enzymes can be devided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non reducing end successively resulting in short end products (Gupta, et al., 2003; Pathania et al., 2017).

Amylase enzymes are among the most important enzymes for biotechnology with great significance, constitute a class of industrial enzymes having approximately 25% of the world enzyme market (Rao et al., 1998). Amylase enzymes have many applications in the textile, food, paper, detergent, pharmaceutical, brewing and distilling industries. Amylase enzymes also have other applications such as in liquefaction and saccharification of starch and in the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins (Pandey et al., 2000; Souza and Magalhaes, 2010). Nowadays, spectrum of applications of amylase enzymes are also extending in many other areas such as analytical chemistry, clinical and medicinal diagnosis eg. diagnosis of acute inflammation of pancreas, macroamylasemia, perforated pelvic ulcer and mumps (Muralikrishna et al., 2005; Anto et al., 2006; Nimkar et al., 2010; Chimata et al., 2010).

Amylase enzymes can be derived from several sources such as plants, animals and microorganisms (Reddy et al., 2003; Pandey et al., 2005). The emzymes from microbial sources are generally used for industrial applications. Amongs microbial sources, fungal sources amylases are very important because amylases of fungal origin was found to be more stable than the bacterial enzyme on a commercial scale (Sanghvi et al., 2011). Major advantage of using fungi for the production of amylase enzyme is the economical bulk production capacity and ease of manipulation. Many species of *Aspergillus* and *Rhizopus* are used as a source of fungal amylases (Pandey et al., 2005). Many non plant pathogenic fungi had been found to be good sources of amylase enzyme (Takahashi et al., 1994; Jin et al., 1999. Abu et al., 2005; Prakusham et al., 2007; Balkan and Ertan., 2007; Gupta et al., 2008; Oyewale, 2010; 2012; 2013; Bhattacharya et al., 2012, Karnwal and Nigam, 2013; Rajasekar, 2013; Saleem and Ebrahim, 2014; Sundarram and Krishnamurthy, 2014; Sujeeta et al., 2017; Ali et al., 2017). Some plant pathogenic fungi are also capable of producing amylase enzyme (Chaurasia, 1992; Raja Brindha, et al., 2011; Thiyam and Sharma, 2013; Chaurasia et al., 2015).

In view of the above and also due to the lack of any information regarding the amylase producing capacity of *Solanum melongena* fruit rot Pathogen *Rhizopus oryzae* work on its enzymological aspects was undertaken in which the effect of different culture conditions on the production of amylase enzyme were evaluated. The aim of this work was to study the effects of culture medium, temperature and pH on the production of amylase enzyme by *Rhizopus oryzae*, in order to increase the level of this enzyme in culture medium by suitable culture conditions.

Materials and Methods:-

Isolation of *microorganism*:-

The Microorganism used in this study was *Rhizopus oryzae* (IMI NO 223116). The *Rhizopus oryzae* was isolated from diseased brinjal fruit and identified as described earlier (Chaurasia et al., 2013, Chaurasia et al., 2017). It was maintained on potato dextrose agar medium slants under refrigeration at 5^0C. The slants were sub-cultured routinely at an intervals of four-five week.

Culture conditions:-

The following culture conditions were undertaken viz., the effect of different culture media, temperature and pH on the production of amylase enzyme were evaluated:

Effect of culture media on amylase production:-

In the present investigation, eight different broth culture media viz., Brown's Dextrose-asparagine-phosphate, Elliot's, Fernando's, Glucose-dox, Glucose-nitrate, Molisch's and Waksman's were used to study their effect on the production of amylase enzyme by *Rhizopus oryzae*. The composition of culture media (g/L) are given in Table 1.

Table 1:- Compsition of culture media used during the course of investigation.

S.No.	Media	Ingredient	Quantities (g)
1	**Brown's**	$MgSO_4.7H_2O$	0.75
		KH_2PO_4	1.25
		Asparagine	2.00
		Dextrose	20.00
		Starch	10.00
		Distilled water	1000 ml
2	**Dextrose-asparagine-phosphate**	Dextrose	30.00
		$MgSO_4.7H_2O$	0.50
		Asparagine	1.00
		KH_2PO_4	1.50
		Distilled water	1000 ml
3	**Elliot's**	Dextrose	5.00
		Asparagine	1.00
		Sodium carbonate	1.06
		$MgSO_4.7H_2O$	0.50
		KH_2PO_4	1.36
		Distilled water	1000 ml
4	**Fernando's**	$MgSO_4.7H_2O$	5.00
		KH_2PO_4	6.80
		Asparagine	5.00
		Glucose	15.00
		Distilled water	1000 ml
5	**Glucose-dox**	$MgSO_4.7H_2O$	0.50
		KH_2PO_4	1.00
		$FeSO_4.7H_2O$	0.01
		$NaNO_3$	2.00
		KCl	0.50
		Glucose	15.00
		Distilled water	1000 ml
6	**Glucose-nitrate**	Glucose	10.00
		$NaNO_3$	1.00
		KH_2PO_4	1.00
		Distilled water	1000 ml
7	**Molisch's**	Sucrose	20.00
		Peptone	10.00
		KH_2PO_4	0.25
		$MgSO_4.7H_2O$	0.25
		Distilled water	1000 ml
8	**Waksman's**	Glucose	10.00
		Peptone	5.00
		KH_2PO_4	1.00
		$MgSO_4.7H_2O$	0.50
		Distilled water	1000 ml

For the production of amylase emzyme, Erlenmayer flasks of 150 ml capacity were used in which 25 ml of the respective broth culture medium (pH 5.0) was poured and autoclaving was done at 15 lb/sq pressure for 20 minutes and cooled at 35^0C. The sterilized Erlenmayer flasks were inoculated with 7.0 mm diameter of mycelial discs taken from the periphery of two days old colony of the test pathogen growing on potato dextrose agar medium. The

Erlenmayer flasks were incubated for 3, 6, 9, 12, 15 and 18 days at 30^0C in incubator. Three replicates were taken in each case. After each incubation period, the culture filtrate of each set was collected and used for enzyme extraction.

(ii) Effect of temperature on amylase production:
The effect of eight different temperatures, i.e., 15, 20, 25, 30, 35, 40, 45 and 50^0C was tried to study their influence on the production of amylase enzyme. Twenty five ml of sterilized Fernando's broth medium (pH 5.0) was poured into 150 ml Erlenmayer flask and inoculated aseptically with 7.0 mm diameter of mycelial disc of the test pathogen from two day old culture and incubated at different temperatures viz., 15, 20, 25, 30, 35, 40, 45 and 50^0C for 9 days. After 9 days of incubation, the culture filtrate of each set was used for enzyme extraction.

(iii) Effect of pH on amylase production:
To study the effect of different pH, the Farnando's broth medium was used. Twenty five ml of Fernando's broth medium was added to each of 150 ml Erlenmeyer flask with different pH values viz., pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. The pH of the broth medium was adjusted with the help of Backman's pH meter by using 1N HCl or 1N NaOH, to get pH value. The flask's were than sterilized at 15 lb/sq pressure for 20 minutes. A 7.0 mm diameter of mycelial disc taken from two days old culture of *Rhizopus oryzae* was inoculated to each of Erlenmayer flask and incubated at 30^0C for 9 days. After incubation, the culture filtrate of each set was used for enzyme extraction. Each set was run in triplicates.

Extraction of amylase:-
The method of enzyme extraction described by Chaurasia, et al., (2015) was used. After desired incubation, fungal mat was removed from the medium and the culture filtrates of *Rhizopus oryzae* were collected in separate flasks by filtration under suction. The culture filtrates thus obtained were centrifuged at 10,000 rpm at 4^0C for 20 minutes. After centrifugation, the clear supernatant liquids obtained decanted and used as the crude enzyme preparations.

Assay of amylase activity:-
Enzyme preparations thus obtained were assayed for the activity of amylase. The activity of amylase was determined by cup plate assay method as described by Chaurasia et al., (2015).

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 (10 mm diameter) was made in the center of each Petridish with the help of cork borer. After this, the bottom of the cavity of each Petridish was sealed by adding two drop of melted agar. 0.2 ml of anzyme extract (culture filtrate) of *Rhizopus oryzae* was added to the cavity with the help of micropipette and incubated at 30^0C temperature. After 24 hours, the Petridishes were treated with Logol's iodine solution (Iodine, 5.0g; Potassium iodide, 10.0g; Distilled water 1000 ml.). After iodine treatment a clear non-blue zone was measured in mm and the activity of amylase expressed after subtracting the diameter of cavity from the diameter of non-blue zone. Each test was run in triplicates. The amylase activity was calculated by the following formula:

$$\mathbf{AA = D-d}$$

Where,

AA = Amylase activity.
D = Diameter of cavity in mm plus diameter of non-blue zone in mm
d = Diameter of cavity in mm.

Results and Discussion:-

[1] Effect of different culture media on amylase production:
The effect of eight different broth culture media viz, Brown's, Dextrose-aspargine-phosphate, Elliot's, Fernando's, Glucose-dox, Glucose-nitrate, Molisch's and Waksman's 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

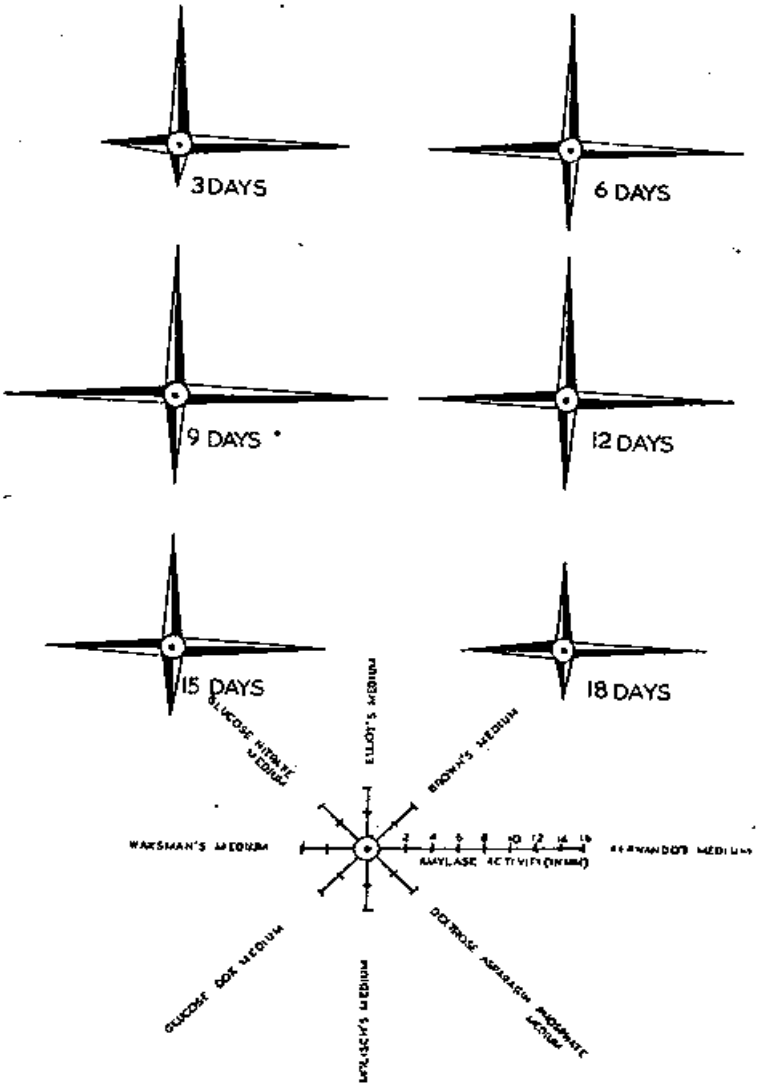
days. The amylase producing capacity of *Rhizopus oryzae* in different culture media is presented in Table 2 and Fig. 1.

Table 2:- Effect of different culture media on the production of amylase enzyme.

Media	amylase activity (width of non-blue Zone in mm)* Days of incubation					
	3	**6**	**9**	**12**	**15**	**18**
Brown's	0.0	0.0	0.0	0.0	0.0	0.0
Dextrose-asparagine-phosphate	0.0	0.0	0.0	0.0	0.0	0.0
Elliot's	10.0	10.5	12.0	10.5	8.0	6.0
Fernando's	12.0	12.5	15.0	12.0	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
Molisch's	2.5	5.0	6.5	5.5	4.5	3.0
Waksman's	5.0	10.0	12.0	10.5	8.5	7.0

***After deducting the cavity of 10.0 mm diameter.**

Fig. 1:- Effect of Different Culture Media on the Production of Amylase



It is clear from the results that *Rhizopus Oryzae* was not capable to produce amylase enzyme in Brown's, Dextrose-asparagine-phosphate, Glucose-dox and Glucose-nitrate media. Therefore, it can be concluded that these media have not favoured the amylase production. It might be due to the depletion of essential nutrients in the culture medium witch created the stressed conditions for pathogen *Rhizopus Oryzae* consequent the inactivation of secretary machinery of the amylase enzyme. Chaurasia et al., (2015) has also reported that Glucose-dox and Glucose-nitrate broth culture media did not support the amylase production as in these culture media *Sclerotium rolfsii* was unable to produce amylase enzyme. Molisch's medium was proved to be poor for amylase production. The poor amylase production on Molisch's medium was either due to the lack of some components in medium which is necessary for fungal growth and enhanced amylase production or due to the presence of repressor substances in media components. In the culture filtrates of Elliot's, Fernando's and Waksman's media an appreciable amount of amylase was detected. Amongst these three culture media, Fernando's medium was found to be the best medium for the maximum production of amylase. Significantly good amount of amylase production of *Sclerotium rolfsii* was also recorded in the culture filtrate of Fernando's medium as has been reported by Chaurasia et al., (2015).

From the above results, it can be concluded that the chemicals incorporated in the media acted differently and thus amylase production obtained in different media were different.

From the results, it was also observed that *Rhizopus oryzae* could be able to synthesize amylase enzyme within a short time, i.e., in three days. The amylase production rapidly increased with the length of incubation period upto 9 days. In 9 days of incubation period, the maximum amylase production was recorded. Further increase in length of incubation period upto 18 days, had no effect on amylase production as seen by the gradual reduction in the amylase enzyme activity. Similar results have also been reported in various fungi by several workars, e.g., *Cephalosporium* species. (Mangallam et al., 1967), *Laphotrichus impullus* (Pathak and Agrawal, 1977), *Myrothecium verrucaria*, *Fusarium equiseti*, *Sporotrichum xylophila* (Pandey and Saxena, 1982), *Sclerotium rolfsii* (Chaurasia et al., 2015). The optimum period for maximum production of amylase was found to be 10 days by Jain (1982) in case of some keratinophilic fungi. It has been also reported that after maximum production of amylase enzyme (maximum incubation time), there was production of other byproducts and a depletion of nutrients. These byproducts inhibited the growth of organisms and hence, enzyme formation (Ali, 1992; Gupta et al., 2008).

From the above results it can be concluded that Fernando's medium and nine days incubation period was found to be the best for the maximum production of amylase enzyme. The enzyme is constitutive clearly shown by their production in good amounts in the medium lacking starch materials.

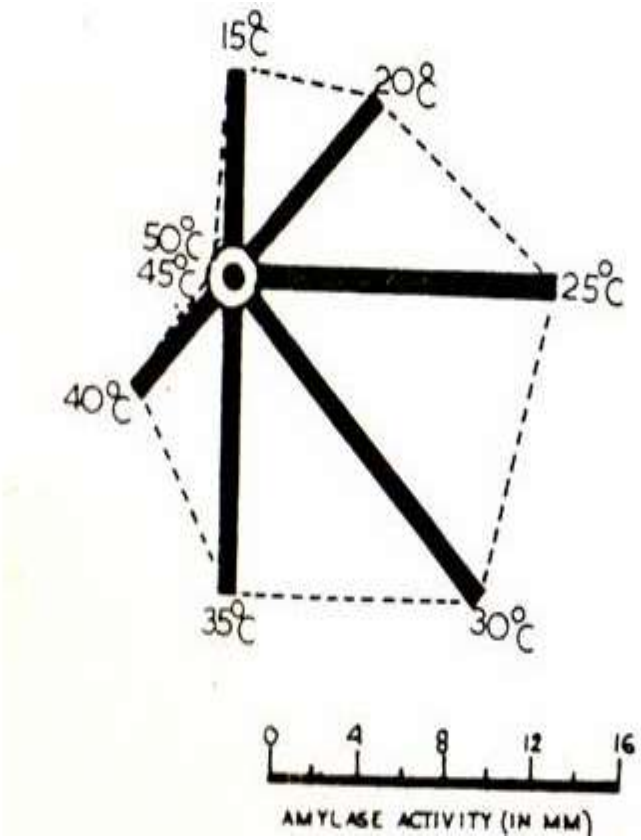
(ii) Effect of different temperature on amylase production:
In order to study the effect of different temperatures on the production of amylase enzyme, the *Rhizopus oryzae* was cultured on Fernando's medium at 15, 20, 25, 30, 35, 40, 45 and 50^0C temperature for 9 days. The results ore presented in Table 3, Fig. 2 and Plate 1.

Table 3:- Effect of different temperatures on the production of amylase enzyme.

Temperature (^{0}C)	amylase activity (width of non-blue Zone in mm)*
15	6.2
20	8.3
25	13.7
30	15.0
35	10.5
40	5.0
45	0.0
50	0.0

***After deducting the cavity of 10.0 mm diameter.**

Fig. 2:- Effect of different temperatures on the production of amylase



It is evident from the Table 2, Fig. 2 and Plate 1 that *Rhizopus oryzae* was able to synthesize amylase enzyme between wide range of temperature, i.e., from 15^0 to 40^0C. Although at 15^0 and 40^0C temperatures, the amylase production was observed very poor. Above 15^0C temperature, the production of amylase enzyme was gradually increased with increase in temperature upto 30^0C. Further increase in temperature upto 40^0C, the amylase production was considerably reduced. In the culture filtrate of 45^0C and 50^0C temperatures, no trace of amylase production was recorded. These results are in agreement with results reported by Adejuwon et al. (2015), who reported an optimal temperature of amylase production to be 30^0C for *Rhizopus arrhizus*. Many workers have also reported that 30^0C was the optimum temperature for amylase production by several fungal genera and species including *Aspergillus flavus* (Mukherjee and Majumdar, 1973), *Penicilium fellutanum* (Ramachandran et al., 2004), *Aspergillus* sp. JGI 12 (Alva, et al, 2007), *Aspergillus niger* (Bhattacharya et al., 2012; Dalal et al., 2014 O*gbonna et. al.,2014)*, *penicillium* sp, *chrysosporium* sp (Ogbonna et al., 2014) and *Sclerotium rolfsii* (Chaurasia et al., 2015).

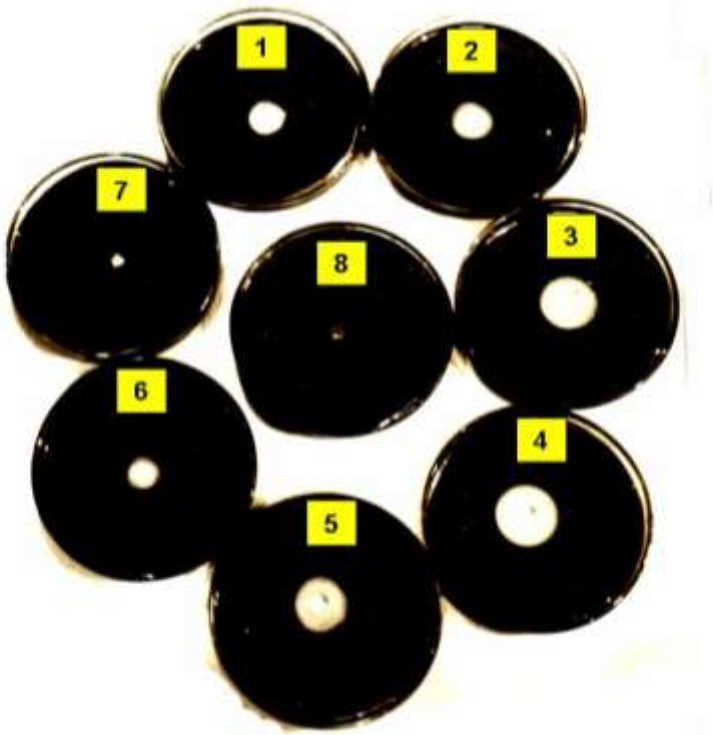


Plate 1:- Showing amylase production at different temperatures (After 9 days of inoculation period)
1. 15^{0}C 2. 20^{0}C 3. 25^{0}C 4. 30^{0}C 5. 35^{0}C 6. 40^{0}C 7. 45^{0}C 8. 50^{0}C

From the above results, it can be concluded that 30^{0}C temperature was found to be the most favourable for the maximum production of amylase enzyme. With further increase in temperature upto 40^{0}C the amylase production gradually decreases. This may be due to the loss of moisture in the substrate which adversely effects the metabolic activities of the microbes leading to reduced growth and decline in amylase production (Prakasham et al., 2007). Higher temperatures above 40^{0}C, i.e. 45^{0}C and 50^{0}C were found to be detrimental for the production of amylase. The detrimental effect in the production of amylase may be due to the lethal effect of *Rhizopus oryzae* at higher temperatures i.e, 45^{0}C and 50^{0}C. These findings clearly indicate the important role of temperatures in the amylase secretion.

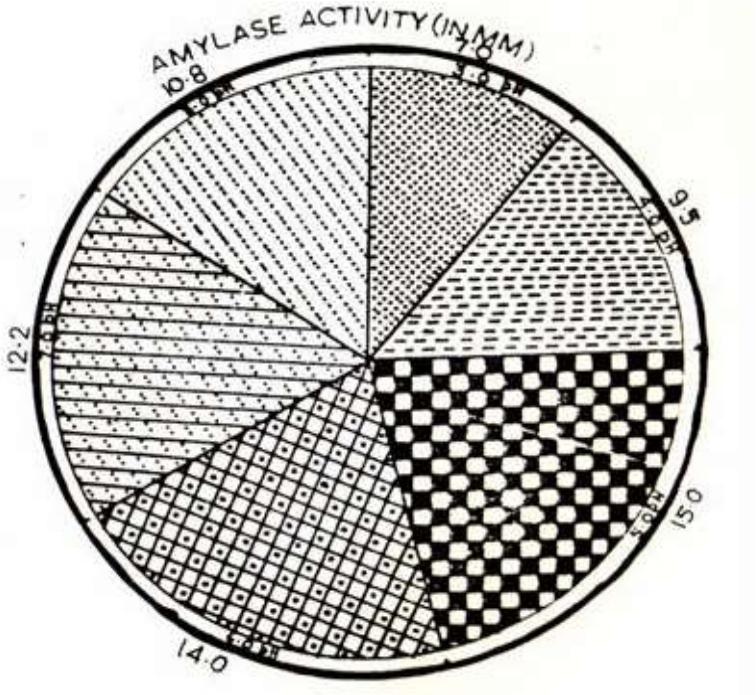
(iii) Effect of different pH on amylase production:-
To study the effect of different hydrogen ion concentration (pH) on the production of amylase enzyme, the *Rhizopus oryzae* was cultured on Fernando's medium having different pH values, viz., 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 at 30^{0}C for 9 days. The results are presented in Table 4, Fig.3 and plate 2.

Table 4:- Effect of different pH on the production of amylase enzyme.

pH	Amilase activity (width of non-blue Zone in mm)*
3.0	7.0
4.0	9.5
5.0	15.0
6.0	14.0
7.0	12.2
8.0	10.8

***After deducting the cavity of 10.0 mm diameter.**

Fig. 3:- Effect of different pH on the production of amylase



Results clearly revealed that *Rhizopus oryzae* was able to produce amylase enzyme on a wide range of pH i.e., from pH 3.0 to 8.0. this suggest that amylase enzyme would be useful in process that require wide range of pH change from slightly acidic to slightly alkaline range from vice-versa. Similar results have also been recorded by Alva et al., (2007) and chaurasia et al. (2015). At extreme acedic pH value i.e., pH 3.0, production of amylase was found to less and further increase in pH value from pH 3.0, the amylase production was gradually increased with increase in pH upto 5.0 which was recorded as optimum pH for maximum production of amylase enzyme. Above pH 5.0, further increase in pH, have no effect but rather resulted in gradual decrease in the production of amylase enzyme. Results indicated that amylase production decrease at above and below optimal pH (pH 5.0). These results are with the findings of Sindhu et al., (2009), who have reported maximum production of amylase at pH 5.0 in case of *Penicillium janthinellum.* Ogbonna et al., (2014) also reported the maximum production of amylase by *Aspergillus niger*, *Penicillium* sp. and *chrysosporium* sp. at pH 5.0, Earlier investigations reported optimum amylase production by *Aspergillus ochraceus* at pH 5.5 (Nahas and Waldemarin, 2002), *Aspergillus awamori* at pH 5.5 (Negi and Banerjee, 2010), *Trichoderma viride* at pH 5.78 (Juwon and Emmanuel, 2012) and *Sclerotium rolfsii* at pH 6.0 (Chaurasia et al., 2015). Mukherjee and Majumdar (1973) while working on *Aspergillus niger* found maximum amylase production at low pH (i.e. pH 3.5). In the present study, the low pH (i.e. pH 3.0) was most unfavourable for amylase production. Others have reported acidic pH optima for amylases from *Aspergillus niger* (Hornandez et al., 2006; Mitieri et al., 2006). Different organisms have different pH optima and decrease or increase in pH on either side of the optimum values results in poor microbial growth (Ramesh and Lonsane (1991).

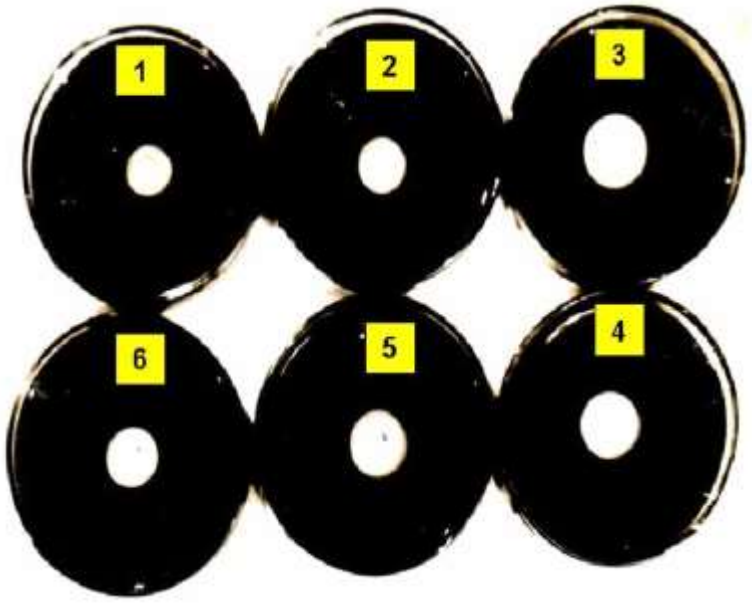


Plate 2:- Showing amylase production at different pH (After 9 days of inoculation period).
1. pH 3.0 2. pH 4.0 3. pH 5.0 4. pH 6.0 5. pH 7.0 6. pH 8.0

From the above results it can be concluded that pH 5.0 was found to be the most favourable for the maximum production of amylase enzyme.

Conclusion:-

Amylases are extensively used in industrial applications like starch modification, food processing and pharmaceutical etc. Amylase producing fungal pathogen was isolated from diseased brinjal fruit and identified as *Rhizopus oryzae* (IMI No.223116). On the basis of above study it may be concluded that amylase enzyme can be produced for industrial purpose from *Rhizopus oryzae* (went & prins Geerl.) grown on Fernando's broth medium (pH 5.0) at 30^0C for 9 days incubation period.

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 textile industries, food industries, paper industries, detergent industries, baking industries, brewing industries, distilling industries and pharmaceutical industries.

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