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# Effect of Incubation Period (with Static and Shaking Condition) on α-Amylase Production from *Aspergillus flavus*

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**Abstract:** Enzymes are act as biocatalyst for a wide variety of chemical reactions. Amylases are extracellular enzymes which can hydrolyse starch to glucose units. Microbial amylases have immense applications in various fields in world market because of their wide application. In the present study, *Aspergillus flavus* fungal species isolated from solid waste dump site soil was used for amylase production to observed the suitable incubation period (3rd day, 5th day, 7th day, 9th day, 11th day and 13th day) and analysed the effect of shaking and static condition for amylase production. The result indicate that after 11th day of incubation at pH 5.5, temperature 27 ±1°C in shaking condition Maximum 47.002 ±1.01 U/ml of α-Amylase was produced and in static 27.105 ±0.80 U/ml of α-Amylase was produced. Compared to both, in shaking condition production of amylase was maximum. It was clearly observed that incubation period and condition play an important role in the production of amylase enzyme.

**Key words:** Amylase Production · *Aspergillus flavus* · Static and Shaking Condition

## INTRODUCTION

Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrin and progressively small polymers composed of glucose unit. The history of amylase began in 1811 when the first starch degrading enzymes was discovered by Kirchhoff. This was followed by several reports of digestive amylase and malt amylases. Ohisson 1930 suggested the classification of starch digestive enzyme in malt α and β amylases according to the anomeric type of sugars produced by the enzyme reaction. In 1831, Erhard Friedrich Leuchs, described the hydrolysis of starch by saliva, due to the presence of an enzyme in saliva, "ptyalin", an amylase [1]. The modern history of enzymes began in 1833, when French chemists Anselme Payen and Jean-François Persoz isolated an amylase complex from germinating barley and named it "diastase [2]. Alpha-Amylase (EC 3.2.1.1) also named as 4-α-D-glucan glucanohydrolase, has found its application in a range of industries including food, brewing, distilling industry, textile, paper pharmaceutical and bioconversion of solid waste etc [3, 4]. Large range of applications is the triggering factor for the industrialization of alpha amylase production. Amylase enzymes account for about 30 % of the world's enzyme production [5-7]. The world market for enzymes remains in excess of $4500 million and increase by 4% annually [6, 8]. With continuously increase in modernization and population, use of enzyme is also increases. But production rate is not as per need of enzyme. So in the present study, fungal species isolated from soil was used for amylase production and observed the suitable incubation period and analysed the effect of shaking and static condition for amylase production.

## MATERIALS AND METHODS

Enzymes are act as biocatalysts and α-amylase is one of the most usable enzyme in industries. Now most of the amylase enzyme is produced from microorganism. Fungi are the major producer of amylase enzyme. So in this work fungus was used for the amylase production in different incubation condition.

**Microorganism:** *Aspergillus flavus* was used in this study for amylase production, which was isolated from soil sample of solid waste dump site, collected from Govt.

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DB Girls PG College Campus of Raipur, Chhattisgarh, India through serial dilution method. Fungus was maintained in potato dextrose agar (PDA) slant at 4°C [9, 10].

**Screening for Amylase Production:** α-Amylase producing fungi was screened by starch hydrolysis test. Fungus was inoculated and grown in Starch Agar Medium (SAM) at 27 ±1°C for 3 to 4 days. After incubation starch hydrolysis test was performed by iodine solution (0.3 % $I_2$ and 1 % KI). Iodine solution was flooded to the SAM plate fungal culture and positive result was showed in clear zone in the SAM medium and negative result having no zone [9, 11].

**Fermentation and Culture Condition:** Production of α-Amylase from fungus was carried out in modified Production Medium [12, 13]. Fungus was firstly grown in Potato Broth (PB) for further use at proper condition. After proper growth of fungus two drops of Tween 80 solution was added into the PB culture and shake the cultured flask. Then 100 µl of cultured broth was inoculated in the Erlenmeyer flask containing 50 ml of Production Medium (g/l), Starch - 5 g, Yeast extract - 5 g, $K_2HPO_4$ - 0.5 g, $MgSO_4$. $7H_2O$ - 0.2 g, $CaCl_2$. $2H_2O$ - 0.1g. Then flasks were incubated at 27 ±1°C at pH 5.5 for different incubation period (3rd day, 5th day, 7th day, 9th day, 11th day and 13th day) [10, 14, 15] in static and shaking condition for amylase production.

**α-Amylase Assay:** Amylase enzyme was assayed by modified iodine assay method [12, 16, 17]. In 0.5 ml of 1 % starch and 0.3 ml of 0.1 M Sodium acetate buffer with 1 ml of distilled water mixture was incubated at 55°C for 10 minutes for equilibrium. Then 0.5 ml of crude enzyme extract was added and allow to reaction for 5 minutes. Then to stop reaction 0.5 ml of 1 M HCl added to the reaction mixture and diluted the mixture volume up to 15 ml by distilled water with adding 0.1 ml of iodine reagent. Then absorbance was taken at 610 nm with the help of Spectrophotometer. One unit of α-amylase is defined as the amount of enzyme which hydrolyzes 0.1 mg of starch in 10 min at 55°C in assay condition.

**Protein Concentration:** Concentration of protein determined by lowry's method [18] using bovine serum albumin (BSA) as standard.

**Statistical Analysis:** α-amylase production was subjected to a test for significant sequential models, which was performed by analysis of variance (ANOVA). All the experiments were performed in triplicates and repeated three times. The samples collected from each replicate were tested for amylase activity. Mean values were analyzed according to Duncan's Multiple Range (DMR) Test compared at 5 % level of significant difference.

## RESULTS

With continuously increase in modernization and population, use of enzyme is also increases. But production rate is not as per need of enzyme. Still many researchers are trying to find out new techniques for the large production of amylase enzyme. So in this study different incubation period and condition was tried to increase the production of amylase enzyme.

**Microorganism and Screening of α-Amylase:** *Aspergillus flavus* fungus was used in this study for production of amylase. *Aspergillus flavus* shows rapid growth, olive to lime green in front with cream colour in reverse, in PDA medium (Fig. 1 and 2)

Amylase production was screened by starch hydrolysis test with iodine reagent. Appearance clear zone indicates the production of amylase and hydrolysis of starch in that area and negative results shows the blue colour in the entire SAM medium [9, 11] (Fig. 3).

**α-Amylase Production:** α-Amylase production was performed in two culture condition Static and Shaking, at pH 5.5 and Temperature 27 ±1°C at different incubation period (3rd day, 5th day, 7th day, 9th day, 11th day and 13th day) with *Aspergillus flavus* and estimated in Unit/ml (U/ml). Production of amylase was increased with increases the incubation period up to 11th day then decreases on further incubation in both the static and shaking condition. Production of amylase was maximum in 11th day of shaking condition 47.002 ±1.01 U/ml compared to static 27.105 ±0.80 U/ml. It was clearly showed that incubation period and condition played an important role in the production of amylase enzyme. (Table 1 and Fig. 4).

**Protein Concentration:** Protein concentration of both the static and shaking crude enzyme extract of different incubation period was estimated by Lowry's method using BSA as standard. Amount of enzyme was related to the concentration of protein. With increases in concentration of protein, the unit amount of enzyme was also increased. But incubation condition was affect the

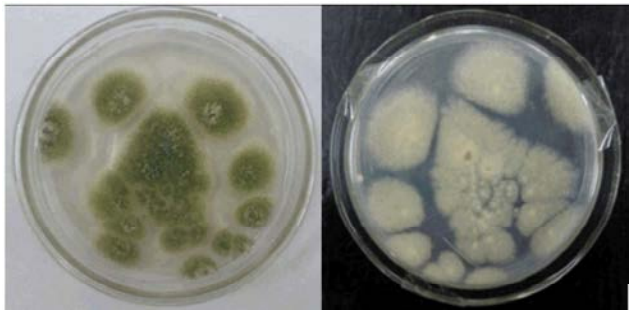

Fig. 1: Colony morphology of *Aspergillus flavus* (Front and Back view in PDA Medium)

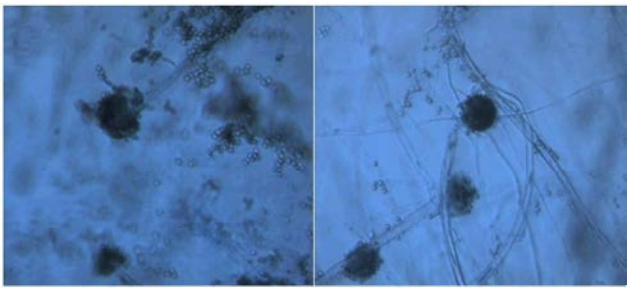

Fig. 2: Microscopic photographs of *Aspergillus flavus*

Fig. 3: α-Amylase screening positive test of *Aspergillus flavus*

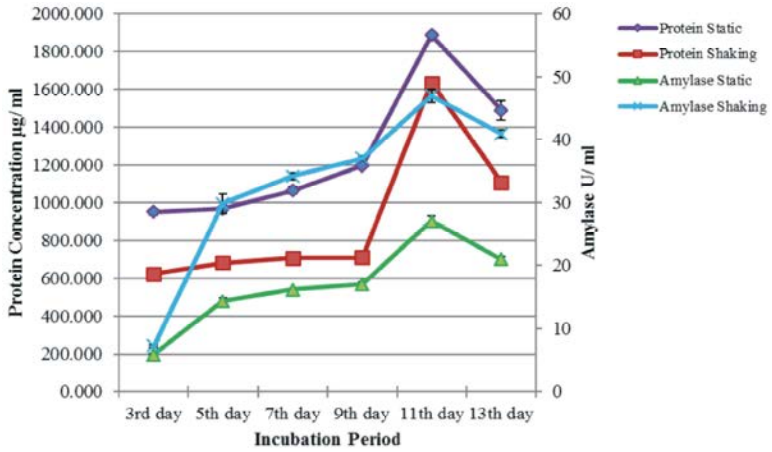


Fig. 4: Production of α-Amylase by *Aspergillus flavus* and concentration of protein in static and shaking condition

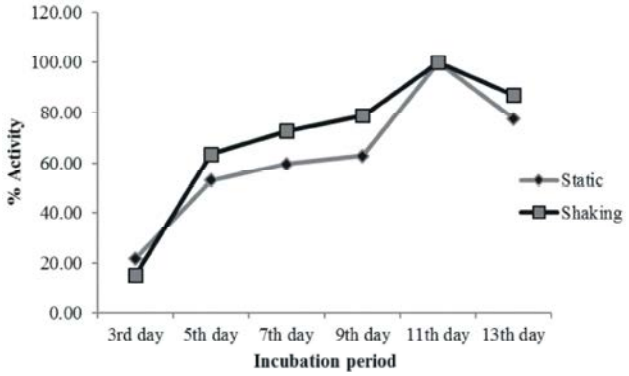


Fig. 5: Activity percentage of amylase enzyme in different incubation condition

Table 1: Production of α-Amylase by *Aspergillus flavus*

| | Incubation 27 ±1°C, pH-5.5 | | |
|---|---|---|---|
| | | Condition | |
| S. no. | Period | Static Mean ±SE U/ml | Shaking Mean ±SE U/ml |
| 1. | 3rd day | 5.949 ±0.12$^{e}$ | 7.265 ±0.21$^{f}$ |
| 2. | 5th day | 14.379 ±0.50$^{d}$ | 29.865 ±1.53$^{e}$ |
| 3. | 7th day | 16.157 ±0.09$^{c}$ | 34.213 ±0.54$^{d}$ |
| 4. | 9th day | 17.029 ±0.38$^{c}$ | 37.107 ±0.34$^{c}$ |
| 5. | 11th day | 27.105 ±0.80$^{a}$ | 47.002 ±1.01$^{a}$ |
| 6. | 13th day | 21.031 ±0.38$^{b}$ | 40.963 ±0.60$^{b}$ |

* ANOVA Static – df = 5, f = 246.06, p < 0.000, LSD (5%) = 1.383
* ANOVA Shaking – df = 5, f = 273.28, p < 0.000, LSD (5%) = 2.568
*Mean followed by similar superscript letters (a, b, c, d, e and f) do not differ significantly at 5% level by Duncan's Multiple Range Test.

Table 2: Estimation of protein concentration on static and shaking condition

| | Incubation 27 ±1°C, pH-5.5 | | |
|---|---|---|---|
| | | Condition | |
| S. no. | Period | Static Mean ±SE µg/ml | Shaking Mean ±SE µg/ml |
| 1. | 3rd day | 951.071 ±15.921$^{e}$ | 623.540 ±18.168$^{d}$ |
| 2. | 5th day | 967.893 ±15.016$^{e}$ | 682.338 ±9.190$^{cd}$ |
| 3. | 7th day | 1063.52 ±19.090$^{d}$ | 706.855 ±3.431$^{c}$ |
| 4. | 9th day | 1196.241 ±5.352$^{c}$ | 708.819 ±35.483$^{c}$ |
| 5. | 11th day | 1890.361 ±10.689$^{a}$ | 1636.169 ±20.866$^{a}$ |
| 6. | 13th day | 1489.491 ±52.633$^{b}$ | 1108.627 ±22.635$^{b}$ |

* ANOVA Static – df = 5, f = 215.13, p < 0.000, LSD (5%) = 77.1
* ANOVA Shaking – df = 5, f = 356.57, p < 0.000, LSD (5%) = 64.55
*Mean followed by similar superscript letters (a, b, c, d and e) do not differ significantly at 5% level by Duncan's Multiple Range Test.

Table 3: Activity percentage (%) of α-Amylase at different incubation period in static and shaking condition

| | Period | | | | | |
|---|---|---|---|---|---|---|
| Condition | 3rd day | 5th day | 7th day | 9th day | 11th day | 13th day |
| Static | 21.95% | 53.05% | 59.61% | 62.83% | 100% | 77.59% |
| Shaking | 15.46% | 63.54% | 72.79% | 78.95% | 100% | 87.15% |

concentration of protein as compared to unit amount production of enzyme in static and shaking condition. In this study concentration of protein was showed maximum in static condition. (Table 2 and Fig. 4).

**Percentage Activity:** In this work percentage activity of amylase enzyme in static and shaking condition at different incubation period was calculated, higher enzyme amount was taken as hundred percentage of relative activity. In the both the condition at 11th day of incubation relative activity was maximum. With increase and decrease of incubation period from 11th day, relative activity was varied. It was observed that incubation period and condition affect the activity of amylase enzyme (Table 3 and Fig. 5).

## DISCUSSION

Some other researchers are also worked with *Aspergillus* species, Akpan *et al*. [19] studied on the production of alpha amylase by *Aspergillus niger* in a cheap solid medium using rice bran and agricultural materials. Sharma and Shukla [20] worked on *Aspergillus fumigatus* and *Chaetomium globosum* isolated from soil sample. Akpan and Adelaja [21] worked with *Aspergillus oryzae* for the production of amylase from rice bran solid medium. Figueira and Hirooka [22] produce amylase from fungi *Fusarium moniliforme* and *Aspergillus flavus*. They found maximum amylase production from *Aspergillus flavus*.

In production of amylase, there are some other workers are also found some relevant results in amylase enzyme production with some fungal sp., Valaparla [23] found maximum amylase activity in Maize starch substrate of 13.59 U/ml and lowest activity in Tapioca starch substrate of 4.66 U/ml at 30°C in shaking condition from *Acremonium sporosulcatum*. Sindhu *et al*. [10] worked on

the different α-amylase production parameters, they found that at pH 5 the production of amylase was maximum. They also worked on incubation period up to 7 days and temperature 30 to 55°C, at 4th day of incubation at 30°C production of amylase was maximum with *Penicillium janthinellum* NCIM 4960. Sharma and Shukla [20]worked on *Aspergillus fumigatus* and *Chaetomium globosum* isolated from soil sample. They found maximum amylase enzyme activity of *Aspergillus fumigatus* in 9 to 11 days of incubation. Yagar *et al*. [24] found the optimum pH and temperature was 5.0 and 40°C respectively from *Aspergillus sclerotiorum*. Balkan and Ertan (2007) produce α-Amylase from *Penicillium chrysogenum* by different agriculture waste products, in their work maximum production of amylase from Wheat Bran (WB) 160 U/ml [16]. Figueira and Hirooka [22] found the highest amylase production, which was at the 10th day of fermentation, from *Fusarium moniliforme* and *Aspergillus flavus*. They found maximum amylase production from *Aspergillus flavus*.

Tiwari *et al*. [25] found 3.07 mg/ml extracellular concentration of protein from *Penicillium rugulosum*. Valaparla [23] found maximum amount of protein from Maize starch was 34 mg by *Acremonium sporosulcatum*. Amutha *et al*. [26] found 0.337 mg/ml of protein at stationary phase from *Bacillus subtilis* KCX006.

Bakri *et al*. [27] studied in the relative activity effect of pH, temperature and incubation time on enzyme activity. They found highest percentage of activity in pH – 6 and the incubation time of enzyme is 60°C and at 50°C 87 % of activity was determined, they found suitable temperature for activity is between 40°C to 60°C. Amutha *et al*. [26] found maximum activity in 10 min. at 50°C. Yagar *et al*. [24] worked on the effect of pH and temperature on percentage activity of enzyme. They found highest percentage of enzyme activity in pH 5 and temperature 40°C.

## CONCLUSION

In this work effect of incubation period in two conditions was analysed and it was found that incubation period and condition plays an important role for the production of amylase. *Aspergillus flavus* was used for the production of α-Amylase at different incubation period in two condition static and shaking at pH 5.5, temperature 27 ±1°C. In both the condition production of amylase was increased with increases in incubation period. In 11th Day of incubation, production of amylase was maximum in both the condition, it was clearly showed that after 11th day of incubation at pH 5.5, temperature 27 ±1°C in shaking condition production of α-Amylase was maximum compared to static condition.

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