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Comparative study of extracellular amylase production by *Aspergillus niger* and *Aspergillus flavus* and effect of temperature and metal ions on enzyme activity

• **Shweta** • **Jyoti Kumari** • **Bhavya Priyadarshini Mishra**
• **Swati**

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Corresponding Author : Swati

Abstract : *A comparative study of production of extracellular amylase was carried out with Aspergillus niger and Aspergillus flavus. Different temperature and pH were provided to the test organism to optimise the enzyme production. Amylase activity was determined using DNS method suggested by Miller (1972). Maximum enzyme activity was observed at temperature 40°C and pH 7.0 for A. niger. The optimum temperature for the activity of amylase produced was obtained at 30°C with optimum pH at 7.0 for A. flavus. The results showed that A. niger is a good producer of extracellular amylase at a relatively higher temperature. This provides an insight that amylase produced would be thermostable in nature. Studies involving the effect of temperature on amylase activity showed that A. niger had maximum amylase activity at 50°C i.e. 0.287 U/ml. A. flavus had an enzyme activity of 0.33 U/ml at 40°C which was recorded as maximum. Thus, amylase produced by A. niger has been found to be more thermostable than that of A. flavus. Effect of metal ions on amylase activity was evaluated. Calcium ions at concentration of 0.1mM has inducing effect on enzyme activity in both the organisms, A. niger (0.268 U/ml) and A. flavus (0.398 U/ml). However concentration of 0.1µM has more inducing effect in case of A. flavus. Magnesium ion has inducing effect to the extent of 0.342 U/ml for A. niger but enzyme activity decreased to the extent of 0.319 U/m in A. flavus. Activity of enzyme amylase from A. flavus (0.425 U/ml) highly increased in presence of Zinc ion at concentration of 0.1µM, however it has slight inducing effect on A. niger (0.319 U/ml). Mercury ion has an inhibitory effect on the amylase activity. Therefore, depending upon the conditions and substrates available, Aspergillus niger and Aspergillus flavus could be utilized for amylase production for industrial purpose.*

Key words: *Enzyme amylase, Aspergillus niger, Aspergillus flavus, temperature, metal ions.*

Shweta
B.Sc. III year, Industrial Microbiology (Hons.),
Session: 2010-2013, Patna Women's College,
Patna University, Patna, Bihar, India

Jyoti Kumari
B.Sc. III year, Industrial Microbiology (Hons.),
Session: 2010-2013, Patna Women's College,
Patna University, Patna, Bihar, India

Bhavya Priyadarshini Mishra
B.Sc. III year, Industrial Microbiology (Hons.),
Session: 2010-2013, Patna Women's College,
Patna University, Patna, Bihar, India

Swati
Assistant Professor, Deptt. of Industrial Microbiology,
Patna Women's College, Bailey Road,
Patna–800 001, Bihar, India
E-mail : swatiswarnima@gmail.com

Introduction:

Enzymes, the most remarkable and highly specialized proteins, have extraordinary catalytic function. The catalyse hundreds of stepwise reactions that degrade nutrient molecules, conserve and transform chemical energy, and help in synthesis of biological macromolecules from simple precursor molecules. Amylases are hydrolytic enzymes that stand out as a class of enzymes which are of useful applications in the brewing, textile, detergent, food and pharmaceutical industries (Varalakshmi *et al.*; 2009). Nowadays, the new potential of using microorganism as biotechnological source of industrially relevant enzymes has attracted interest in exploration of extracellular enzymatic activities in several microorganisms (Bhimba *et al.*; 2011). Amylases function by the breakdown or hydrolysis of starch into reducing fermentable sugars, mainly maltose, glucose and reducing non fermentable or slowly fermentable dextrins(Oyeleke *et al.*; 2010). Amylase has been industrially produced from several fungi, yeasts, bacteria and actinomycetes. Enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey *et al.*; 2000).

Moulds of genus *Aspergillus* are especially useful for producing amylase (Khan and Yadav, 2011, Ileasanmi and Oluwaseum, 2012, Bhardwaj *et al.*; 2012,). Present study has been focused on production of amylase by *Aspergillus niger* and *Aspergillus flavus*. Two important growth factors temperature and pH have been found to influence enzyme activity to a considerable extent. Metal ions have also been observed to have some significant effects on the amylase activity (Sudha, 2012) and hence it has been evaluated on this test system. This has yielded fruitful information. Therefore, the aim of the research project was of comparative nature regarding amylase production by *Aspergillus niger* and *Aspergillus flavus* and has been described.

Materials and Methods:

Collection of sample : The pure fungal strains of *Aspergillus flavus* and *Aspergillus niger* were procured from department of Industrial Microbiology, Patna Women's College.

Screening for amylase production : The method of Bhimba *et al*, 2011 was followed. Fungal strains were streaked on Starch Agar medium. The plates were incubated for 5 days at 37°C.The fungal colonies obtained on the plates were flooded with 1% iodine in 2% Potassium iodide. The formation of clear zone was observed which demonstrated amylase activity.

Production of amylase (submerged fermentation) : The method of Bhimba *et al*, 2011 was followed. The cultures of amylase producing strains were inoculated in 30 ml of production medium in conical flasks of 250 ml (Borosil glass). The cultures were incubated on a gyratory shaker having a speed of 120rpm at a temperature of 27°C for 72hrs.

Optimization of culture conditions for enzyme production : Effect of temperature and pH on amylase producing cultures was determined at different temperatures (20, 30, 40, 50 and 60°C) and pH (4, 5, 6, 7 and 8).

Extraction of amylase : Production media containing amylase produced by fungal culture was centrifuged (Remi) at 6000 rpm for 15 minutes as suggested by Bhimba *et al*, 2011 . The supernatant obtained was filtered through Whattmann No. 1 filter paper. The filtrate contained the crude extract of enzyme- Amylase.

Preparation of reagents : Production medium was prepared by adding soluble starch 15g/L, lactose 10g/L, ammonium sulphate 5g/L , calcium chloride 2g/L and sodium chloride 2g/L. 1% iodine was prepared by adding 1g of iodine to 100ml of 2% potassium iodide, which was prepared by adding 2g of potassium iodide in 100ml of distilled water. 1% of soluble starch was prepared by adding 1g of soluble starch in 100ml of

distilled water. For citrate buffer, 9.5ml of 0.1 M citric acid and 41.5 ml of 0.1 M sodium citrate was added to 50 ml of distilled water.

Enzyme Assay :

Assay at different temperature: 1ml of enzyme was extracted by centrifugation in centrifuge at 6000 rpm for 15 minutes and added to 1 ml of 1% soluble starch in citrate buffer. The solution was incubated in water bath at 25^{0}C, 30^{0}C, 40^{0}C, 50^{0}C and 60^{0}C separately for 20 minutes. The reaction was terminated by adding 2 ml of DNS reagent (Miller, 1972). The solution obtained was boiled at 80^{0}C for 5 minutes in water bath. After cooling the solution, 20 ml of distilled water was added to the samples and absorbance was read at 540 nm using spectrophotometer (Thermo). Blank each for *Aspergillus flavus* and *Aspergillus niger* was prepared consisting of 2 ml of enzyme extract which was boiled for 20 minutes (Varalakshmi *et a.;l* 2009).Starch solution was added to the blank and treated with the same reagents as the experimental tubes. Control was prepared without the enzyme extract and the above procedure was followed as it was for experimental tubes.

Assay using different metal ions: To, 01 ml of enzyme extract and 01 ml of chloride salt solutions of Ca^{2+}, Zn^{2+}, Hg^{2+} and Mg^{2+} was mixed with 01 ml of 1% soluble starch in citrate buffer in separate culture tubes. The relative enzyme activity was measured under standard assay conditions at 37^{0}C n pH 8.3.

Result and Discussion:

Screening for amylase production by the test organisms : Both the *Aspergillus* species viz, *A. niger* and *A. flavus* were evaluated for production of amylase by starch hydrolysis. When starch agar medium was inoculated with the organism and subsequently flooded with iodine solution, the clear zone could be observed around the microbial growth which indicated the production of amylase by the organisms. Clear zone was more prominent and large in case of *A. niger* as compared to *A. flavus*. Hence it can be suggested that *A. niger* has greater capacity to produce amylase compared to *A. flavus*.

Production of amylase under submerged condition : Submerged fermentation was carried out in the 250 ml Erlenmeyer flasks by taking 100 ml of amylase production medium under shake flask conditions for definite incubation periods. Fungal mycelia were visible after 36- 48 hours of incubation. After complete incubation period fungal mycelial mat was observed in broth inoculated with *A. niger* and *A. flavus.*

Effect of pH on amylase production : pH is an important factor that determines the morphology and growth of microorganisms as they are quite sensitive to this in the medium. Earlier studies (Bhimba *et al*, 2011) have revealed that fungi prefer slightly acidic pH. *A. niger* and *A. flavus* were inoculated into media with different pH ranging from 4-8 and the enzyme was extracted and the specific activities of the amylase produced at different pH were recorded and this has been shown in figure 1.

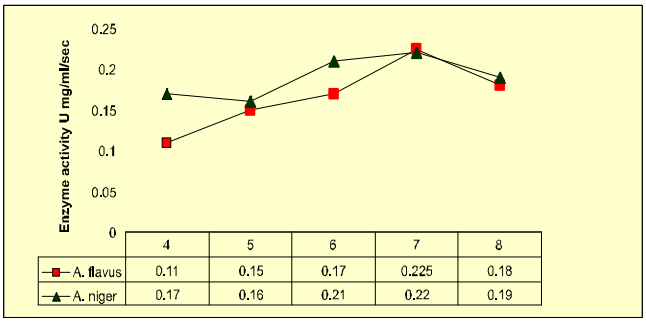

Fig 1: Effect of pH on production of amylase from *Aspergillus niger and Aspergillus flavus*

From the data presented graphically in Fig 1 it appears that *A. flavus* had an enzyme activity of 0.11mg/ml/sec and 0.231 mg/ml/sec at pH 4 and 5,

respectively. It had an enzyme activity of 0.17 mg/ml/sec at pH 6. As the pH of the broth increased to 7, the enzyme activity sharply increased to the extent of 0.225 mg/ml/sec. But further increase in pH of medium decreased the enzyme activity to a value of 0.18 mg/ml/sec. Optimum amylase activity was recorded at pH 7, which reached a peak (0.225 mg/ml/sec). In case of *A. niger*, it had an enzyme activity of 0.17 mg/ml/sec and 0.16 mg/ml/sec at pH 4 and 5, respectively. At pH 6 there was sharp increase in enzyme activity to the extent of 0.21mg/ml/sec. There was slight increase in enzyme activity 0.22 mg/ml/sec at pH 7. This was followed by a decrease in enzyme activity at pH 8 i.e. 0.19 mg/ml/sec. Thus, optimum amylase activity was recorded at pH 7, which reached a peak (0.22 mg/ml/sec). In another set of experiment, effect of temperature on enzyme production was monitored.

Effect of Temperature on amylase production: Test organisms were grown at 20°C, 30°C, 40°C, 50°C and 60°C under liquid shake condition (Bhimba *et al*, 2011). The enzyme was extracted and the specific activities of the amylase produced at different temperatures were recorded and has been described. (Fig 2)

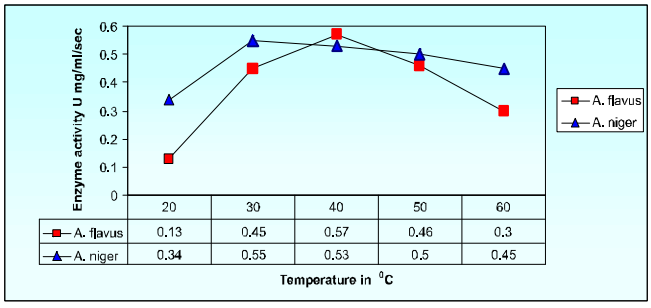

Fig 2: Specific activities of the amylase produced at different temperatures

Fig 2 indicates that *A. flavus* had an enzyme activity of 0.13 mg/ml/sec at 20°C. As the temperature of medium increased to 30°C, the enzyme activity sharply increased to a value of 0.45 mg/ml/sec.This was followed by further increase in enzyme activity at 40°C to the extent of 0.57 mg/ml/sec. However, further increase in temperature decreased the enzyme activity to the value of 0.46 mg/ml/sec at 50°C and 0.30 mg/ml/sec at 60°C. In case of *A. niger*, it had an enzyme activity of 0.34 mg/ml/sec at 20°C and increased to 0.55 mg/ml/sec at 30°C. As the temperature increased to 40°C, the enzyme activity decreased to 0.53 mg/ml/sec. This was followed by further decrease in enzyme activity at 50°C and 60°C i.e. 0.50 mg/ml/sec and 0.45 mg/ml/sec, respectively. Similarly the enzyme activity was also evaluated.

Effect of temperature on amylase activity : The effect of temperature on crude enzyme activity was measured at pH 6.0 over a temperature range of 25°C – 60°C (Figure 3). *A. flavus* had an enzyme activity of 0.288 U/ml and 0.231 U/ml at 25°C and 30°C, respectively. It had an enzyme activity of 0.33 U/ml at 40°C. As the temperature increased to 50°C, the enzyme activity decreased (0.254 U/ml). This was followed by a slight increase in enzyme activity at 60°C (0.263 U/ml). Optimum amylase activity was recorded at 40°C, which reached a peak (0.33 U/ml). In case of *A. niger*, it had an enzyme activity of 0.199 U/ml and 0.254 U/ml at 25°C and 30°C, respectively. It had an enzyme activity of 0.231 U/ml at 40°C. As the temperature increased to 50°C, the enzyme activity increased (0.287 U/ml). This was followed by decrease in enzyme activity at 60°C i.e. 0.263 U/ml. Optimum amylase activity was recorded at 50°C, which reached a peak of 0.287 U/ml.

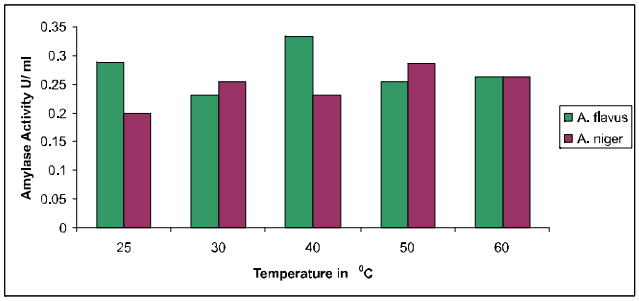

Fig 3: Specific activities of the amylase enzyme obtained from *A. flavus* and A. *niger* at different temperatures

Review of literature pertaining to such studies has revealed that metal ions play a vital role in enzyme activity. In order to have an insight regarding this knowledge, experiments were designed and carried out to evaluate the role of metal ions on production of enzyme amylase on *A. niger* and *A. flavus*.

Effect of metal ions concentration on amylase activity : Amylase is a metalloenzyme which contains at least one activating Ca^{2+} ion. The affinity of Ca^{2+} to amylase is much stronger than that of other ions (Gupta *et al*., 2003). Enhancement of amylase activity such as Mg^{2+}, Ca^{2+} and Zn^{2+} ions could be based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acid (Linden et al., 2003). It was found that metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme. Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, *etc*. was reported to increase a-amylase activity of an alkaliphilic *Bacillus* sp. ANT-6 (Pandey *et al*., 2000; Burhan *et al*., 2003). The stabilizing effect of Ca^{2+} on thermo stability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus, causing the adoption of a compact structure (Goyal *et al*., 2005).

Calcium ion has positive effect on enzyme activity in both the organisms, *A. niger* (0.268 U/ml) and *A. flavus* (0.398 U/ml). However, it has more positive effect in case of *A. flavus.* Magnesium ion has positive effect for *A. niger* (0.342 U/ml) but enzyme activity decreased in case of *A. flavus* (0.319 U/ml). Activity of amylase enzyme from *A. flavus* (0.425 U/ml) highly increased with Zinc ion, however it has positive effect on *A. niger* (0.319 U/ml) as well, but enzyme activity was less. Hg^{2+} has an inhibitory effect on the amylase activity. There was no colour development at the end of reaction but precipitate was obtained. This happened in both the cases i.e. amylase obtained from both the fungal species. No activity was observed in tube where $HgCl_2$ was added. The result has been graphically shown in figure 4.

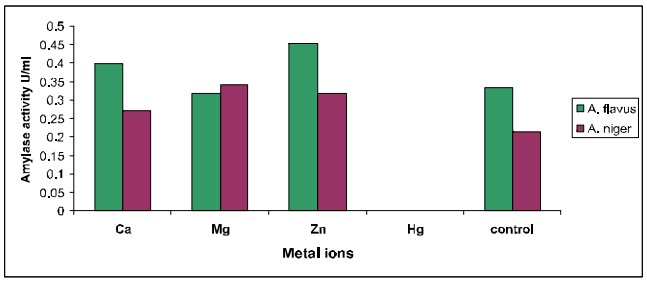

Fig 4: Specific activities of the amylase enzyme obtained from *A. flavus* and A. *niger* with different metal ions.

On review (Fig 4) it appears metal ions (except Hg) have inducing effect on *A flavus* much more than *A. niger*. This finding is in consonance with the earlier findings. (Gupta, 2003; Linden *et al* 2003)

Conclusion :

Our study demonstrated the effect of different temperatures and metal ions on *Aspergillus niger* and *Aspergillus flavus*. Temperature has significant effect on amylase activity because it affects the three dimensional structure of amylase. *A. niger* and *A. flavus* show their maximum activity at different temperatures. *Aspergillus niger* has a slightly more efficiency for amylase production than that of *Aspergillus flavus.* The amylase produced by *Aspergillus niger* is more thermostable than that of *Aspergillus flavus*. As in case of any other enzyme, amylase is also pH sensitive i.e. it can work efficiently at only a particular pH. In our study, pH 7 was preferred as shown by optimization results. In our study, Ca^{2+} and Zn^{2+} were shown to have positive effects on both the species, however, *Aspergillus flavus* was more positively affected than *Aspergillus niger.* In case of Mg^{2+}, the effect on *Aspergillus flavus* was not that much pronounced whereas *Aspergillus niger* was positively affected. Amylase production by both the species showed a total negative effect i.e. inhibitory effect of Hg^{2+} ion. Therefore, depending upon the conditions and substrates available, *Aspergillus niger* and *Aspergillus flavus*

could be utilized for amylase production. As for instance if the readily available media has Mg^{2+}, use of *Aspergillus niger* would be a better option. This also goes for the temperature and pH conditions.

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