International Journal of Biology Research
www.biologyjournal.in
ISSN: 2455-6548
Received: 19-04-2025, Accepted: 04-05-2025, Published: 30-05-2025
Volume 10, Issue 2, 2025, Page No. 32-38

Production of amylase from *Aspergillus Niger* utilizing different agrowaste

Barate DL, Satote PP
Department of Microbiology, Shri Shivaji College, of Arts, Commerce & Science, Akola, Maharashtra, India

Abstract
Agrowaste utilization with submerged liquid fermentation (SmF) holds tremendous potential for the production of the enzyme amylase by *Aspergillus niger*. In the study 13 fungal isolates were screened for alpha amylase production and the isolate A-2 later utilized for production study. Growth of *Aspergillus niger* was found to be optimum at room temperature and pH 6.2. Submerged liquid fermentation was carried out using different substrates namely Wheat bran and Mustard oil cake. Amylase produced using various substrate was having good activity but mustard oil cake as a substrate was found best substrate for production as activity of enzyme was maximum this was followed by 1.85U/ml with wheat bran (1.65 U/ml) and starch (1.35 U/ml).

Keywords: Agrowaste, amylase, *Aspergillus niger*

Introduction

In recent years, the utilization of agro-industrial residues for microbial enzyme production has gained significant attention due to its economic and environmental benefits. Agro-wastes such as wheat bran, sugarcane bagasse, and mustard oil cake are abundant by-products of agricultural and food processing industries, often discarded as waste (Balkan & Ertan, 2007; Bhargav *et al*., 2008) [2, 3]. These residues, rich in carbohydrates, proteins, and essential nutrients, serve as ideal substrates for microbial fermentation, supporting the growth and metabolism of enzyme-producing microorganisms (Ramachandran *et al*., 2004) [9]. Amylase is an industrially significant enzyme widely used in food, fermentation, textile, pharmaceutical, and biofuel industries (Pandey *et al*., 1999; Nigam & Singh, 1995) [8]. It plays a crucial role in starch hydrolysis, converting complex polysaccharides into fermentable sugars. Among microbial sources, filamentous fungi, particularly *Aspergillus spp*., have been extensively studied due to their high amylase yields and extracellular enzyme secretion capabilities (Bhargav *et al*., 2008) [3]. *Aspergillus niger* is one of the most efficient amylase producers and has been widely employed in industrial fermentation processes (Sun *et al*., 2010) [12].

Fermentation techniques such as submerged fermentation (SmF) and solid-state fermentation (SSF) are commonly used for amylase production, each offering unique advantages. SmF allows for controlled conditions and high enzyme yields, where as SSF provides a cost-effective approach using solid agro-residues with minimal processing requirements (Sandhya *et al*., 2005; Hixson & Gaden, 1950). The selection of appropriate agro-waste substrates, along with optimized fermentation conditions, plays a critical role in maximizing amylase production efficiency.

The present study aims to evaluate the potential of different agro-waste materials in enhancing amylase production from *Aspergillus spp*. under controlled fermentation conditions. By optimizing substrate composition and process parameters, this research contributes to the development of sustainable and economically viable enzyme production strategies, reducing dependency on synthetic raw materials while promoting environmental sustainability (Sajjad & Choudhry, 2012) [10].

Materials and methods

1. Isolation of *Aspergillus* niger

A piece of bread was kept in a moist condition at room temperature in dark for 2 days. The bread sample was serially diluted and different dilutions were plated on potato dextrose agar (PDA). The Petri plates were incubated at room temperature for 4 days. Fungal cultures were observed on PDA medium. Different fungal cultures were selected and sub cultured on PDA slants. All the 13 fungal strains were subjected to lactophenol cotton blue staining for studying the morphology. All the fungal cultures were confirmed as *Aspergillus niger* by studying the morphology and the spore colour.

2. Lactophenol cotton blue staining

A loop full of fungal cultures were placed on a clean glass slide, a drop of lactophenol cotton blue stain was then mixed with the culture. A clean coverslip was placed over the culture to avoid air bubbles and viewed under the microscope (45 X) The blue-stained structures allow easy differentiation of fungal species and the morphology of *Aspergillus niger* was observed.

3. Determination of amylase activity

All the *Aspergillus niger* isolates were tested for amylase production by Starch hydrolysis. All the 13 isolates were streaked centrally on sterile solidified Starch agar plates the plates were incubated at 28°c for 48 hours at room temperature then all the plates were flooded with iodine solution, the zone of clearance around the microbial growth indicated the production of amylase. On the basis of area of clearance, the isolate no. 2 (A-2) out of the 13 isolates were selected for further studies on amylase production.

4. Fermentation medium

Production of amylase was carried out by submerged liquid fermentation using the substrates of zero cost namely Wheat

bran, Mustard oil cake, Sugarcane bagasse. For Fermentation 10 gm of powdered Wheat bran, Mustard oil cake and sugarcane bagasse were taken in 250 ml flasks and moistened with nearly 200 ml of medium containing the following in gm/l (0.8 g NaCl, 0.8 g KCl, 0.1 g CaCl2, 2.0 g Na2HPO4, 0.2 MgSO4, 0.1 g FeSO4, 8.0 g Glucose, 2.0 g NH4Cl) a flask without substrate, having Starch soluble powder was also maintained for comparison as standard. Flask were autoclaved, cooled to room temperature, inoculated with the 48-hour old grown culture of showing maximum hydrolysis during screening and incubated at 28°c for 5 days.

5. Enzyme Extraction

After Fermentation, the contents of the flask are filtered using a Whatman No. 44 filter paper followed by filtration through a muslin cloth. Then the filtrate is centrifuged at 10000 rpm for 10 min and the supernatant was used as the source of enzyme

5.1. Enzyme assay

Amylase activity was determined by the dinitrosalicylic method using soluble starch as a substrate. The reaction mixture containing of 1% substrate in 0.1M phosphate buffer, it was kept for 20 mins in incubator at 37°C for activation of enzyme then enzyme was added and again kept it in incubator for 30 mins at 37°C for incubation. The reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid solution followed by heating in a boiling water bath for 10 minutes and cooling at room temperature and then 8ml of distilled water was added. Absorbance of each solutions was measured at 540nm by UV-Visible Spectrophotometer. The enzyme activity was measured according to DNSA method based on the standard curve of sugar.

5.2. Protein assay

The total protein concentration of cell free filtrate was determined by the lowry method using bovin serum albumin as a standard. The following protocols has been followed for determination of proteins.

Table 1: Protocol for determination of standard curve of Bovine serum albumin (BSA

t	Reagent	Blank	1	2	3	4	5
	Std. Protein Solution (0.2mg/ml)	0.0ml	0.2ml	0.4ml	0.6ml	0.8ml	1.0ml
	NaOH (0.1 N)	1.0ml	0.8ml	0.6ml	0.4ml	0.2ml	0.0ml
	Alkaline CuSO4 Lowery Solution	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml
Allow to stand for 10 min at room temperature							
4.	Folin – Ciocalteu's reagent	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
Allow to stand for 30 min and read at 660 nm							
5.	Concentration of protein (ug/ml)	0	40	80	120	160	200

6. Effect of various parameters on enzyme activity There are various factors affecting the enzyme production are pH, temperature, time, nitrogen and carbon sources, metal ions.

6.1. Effect of Temperature: The effect of temperature on amylase production by the *Aspergillus niger* was determined by growing it in assay media using the different temperature of room temperature, 37°C, 40°C. The optimum temperature was found out by inoculating the *Aspergillus niger* culture in the optimized media and incubating it at different temperature. The enzyme Assay was done after 72 hrs by taking optical density at 540 nm.

6.2. Effect of pH: To determine the optimum pH for amylase production, the *Aspergillus niger* culture was inoculated in optimized medium adjusted to pH 5, 7 and 9. Then it was incubated at room temperature for 72 hrs. The media pH was adjusted with 0.1N NaOH or 0.1N HCL. After the incubation, the cultures were harvested, centrifuged and supernatant was used for amylase assay. Optical density was observed at 540 nm, for optimum pH.

6.3. Effect of Metal ion: To determine the effect of metal ions that act as activators or inhibitors used MgSO4 and CuSo4 metal ion. These metal ion is added in media then culture inoculation was done and keep it for 72 hours at room temperature. After the incubation period, the medium was harvested, centrifuged and supernatant was used for amylase assay. Optical density was observed at 540 nm.

6.4. Effect of Time: The effect of time on amylase production by the *Aspergillus niger* was determined by growing it in assay media using the different time ranges after 1 day, after 2 days, 3 day and 4 days. The optimum time period was found out by inoculating the *Aspergillus niger* in the optimized media and incubating it at different time. The amylase assay was done after the respective time period by taking Optical density at 540 nm.

6.5. Effect of carbon sources: To study the efficacy of various carbon sources, the medium was supplemented independently with different carbon sources. The glucose, sucrose and lactose were utilized to observe the effect with 1% concentrations. The test medium supplemented with the required concentration of carbon sources was sterilized, inoculated with test organism and incubated at 37°C for required time. After the incubation, culture was centrifuged and supernatant was used for amylase assay. Optical density was observed at 540 nm.

6.6. Effect of Nitrogen sources: The effect of nitrogen sources on amylase production was studied with different concentrations of nitrogen sources like ammonium chloride, ammonium sulphate, and sodium nitrate. Each source was tested at 1% concentration. The production medium supplemented with required

concentration of nitrogen source was sterilized and inoculated and kept for incubation. After incubation the supernatant was used for amylase activity by checking Optical density at 540 nm.

Result and discussion

1. Screening of amylase producing *Aspergillus* niger

The table no. 2 presents data on the amylase production capability of 13 different isolates, labeled A-1 to A-13. The isolates were categorized based on their amylase production efficiency. Among them, Isolate A-2 demostrates the highest amylase production with an "Excellent" **(+++)** rating. Isolates A-1, A-5, A-8, and A- 11 show "Good" **(++)** production, while the remaining was isolates exhibit "Satisfied" (+) levels. This classification aids in identifying the most efficient amylase- producing was isolate.

2. Production of amylase

The medium was used for the production of enzyme was starch broth and different substrates like wheat bran, mustard oil cake it provides the nutrient required for growth of organiasm. The temperature was maintained at 28°C and the time period was provided of 72 hrs. The cell free supernatant was seperated by centrifugation at 5000 rpm for 20 min and used as crude enzyme. The different optimization parameters were studied.

3. Protein estimation

3.1. Standard graph of bovin serum albumin (BSA) for protein estimation: For the determination of concentration of protein in the crude enzyme sample, A standard graph was plotted with the known concentration of standard protein.

3.2. Protein estimation for crude enzyme: Protein concentration from the crude enzyme sample was determined by folin-Lowry method. The protein concentration was found in the cell free supernatant of different substrate was found for starch it was 104 mg/ml, for wheat bran it was 55 mg/ml and for mustard oil cake it was found to be 68 mg/ml.

3.3. Enzyme assay: For the determination of concentration of amylase in crude enzyme sample, the standard graph was plotted with the known concentration of standard maltose. Amylase concentration for the crude enzyme sample was determined by Sumner's method. From the standard graph the activity of crude enzyme was determined for different substrates was found to be for Starch it was 1.35 U/ml, for wheat bran it was 1.65 /ml and for Mustard oil cake it was 1.85 U/ml.

4. Effect of various parameters on production of amylase enzyme

4.1. Effect of temperature on enzyme production:

Fig no.1 shows effect of different temperatures on amylase production. It compares three conditions: Room Temperature (R.T), 37°C, and 40°C, using wheat bran and mustard oil cake as substrates. At R.T, mustard oil cake exhibits the highest amylase activity (0.9 U/ml), followed by wheat bran (0.75 U/ml), and the lowest activity was seen in the starch (0.6 U/ml). As the temperature increases to 37°C, amylase activity declines for all substrates, with mustard oil cake maintaining the highest activity (0.4 U/ml), wheat bran showing moderate activity (0.3 U/ml), and the starch having minimal activity (0.1 U/ml). At 40°C, the enzyme activity further decreases, with mustard oil cake and wheat bran showing similar values (0.25 U/ml), while the starch remains the lowest (0.1 U/ml). Overall, the data suggest that amylase production was optimal at Room Temperature, particularly when using mustard oil cake, while higher temperatures negatively impact enzyme activity.

In other studies, it was reported that best enzyme production in *A. niger* was at room temperature [Varalakshmi *et al.,* 2009] [14, 15] and reported 30°C to be the best for enzyme production by *A. niger* [Kathiresan and Manivannanm, 2006] [7]. Thwas shows that the enzyme production was greatly affected by temperature. The great yield temperature for enzyme production were between 30°VC-37°C. [Ueno *et al.,* 1987 [13]; Kandu *et al.,* 1973.

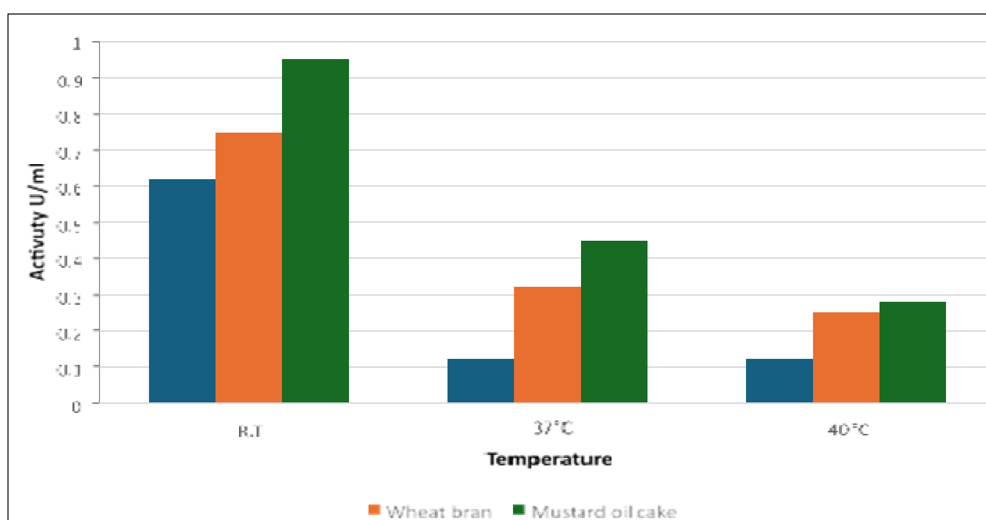

Fig 1: Effect of various temperature on the production of Amylase

4.2. Effect of pH on enzyme production

Fig no.2 shows effect of pH on amylase enzyme production, measured in Activity U/ml. Three different substrates are used: starch, wheat bran, and mustard oil cake. At pH 5**,** amylase production was highest across all substrates, with mustard oil cake showing the maximum activity (0.95

U/ml), followed by wheat bran (0.85 U/ml) and starch (0.6 U/ml). At pH 7, enzyme activity significantly decreases for all substrates, with mustard oil cake maintaining the highest activity (0.35 U/ml), followed by wheat bran (0.3 U/ml) and starch (0.2 U/ml). At pH 9, amylase activity was minimal across all substrates, with values close to 0. U/ml or lower**.** Overall, the data indicate that pH 5 was 1he optimal pH for amylase production**,** while neutral (pH 7) and alkaline (pH 9) conditions result in reduced enzyme activity. Mustard oil cake conswastently yields the highest amylase activity across all pH levels but maximum yield was at pH 5. This was was an accurance with the studies of [Gupta *et al.,* 2008] who also reported the maximum yield of amylase was at pH 5.5. Other studies have also reported that acidic pH supports maximum production of amylase from *Aspergillus* niger. [Hernandes *et al.,* 2006; Mitieri *et al.,* 2006].

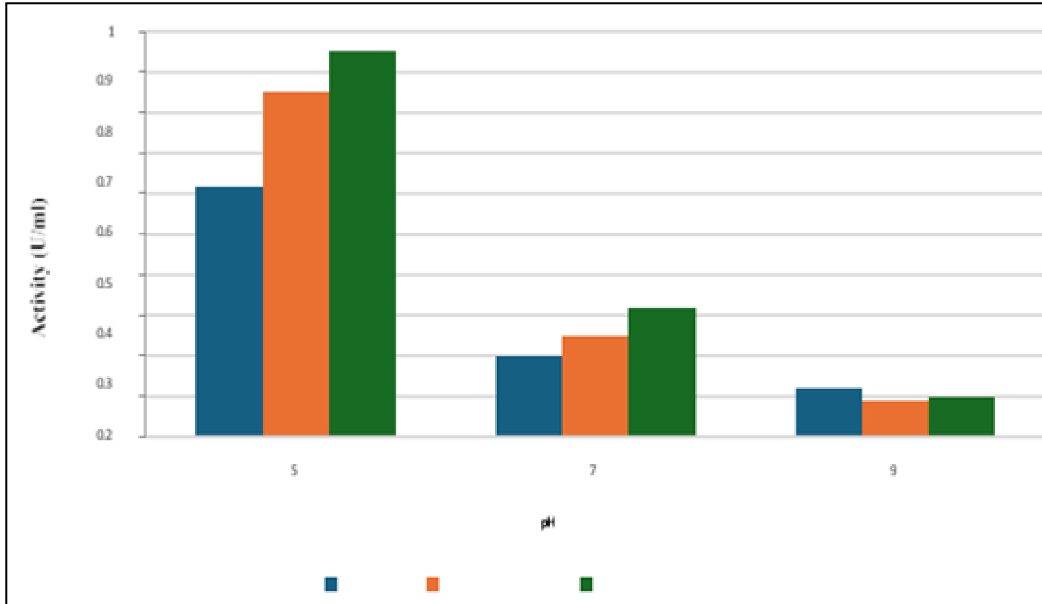

Fig 2: Effect of pH on production of amylase enzyme

4.3. Effect of metal ion on enzyme production

Fig no.3 shows effect of different metal ions ($MgSO_4$ and $CuSO_4$) on the production of amylase enzyme, measured in Activity U/ml. Three different substrates are compared: starch, wheat bran, and mustard oil cake. For $MgSO_4$**,** wheat bran exhibits the highest amylase activity (0.95 U/ml), followed by starch (0.85 U/ml), while mustard oil cake shows the lowest activity (0.65 U/ml). For $CuSO_4$**,** mustard oil cake displays the highest amylase activity (0.85 U/ml), while wheat bran (0.75 U/ml) and starch (0.65 U/ml) show slightly lower values.

Overall, the data suggest that $MgSO_4$ enhances amylase production more in wheat bran and starch**,** whereas $CuSO_4$ was more effective for mustard oil cake**.** The choice of metal ion influences enzyme production depending on the substrate used.

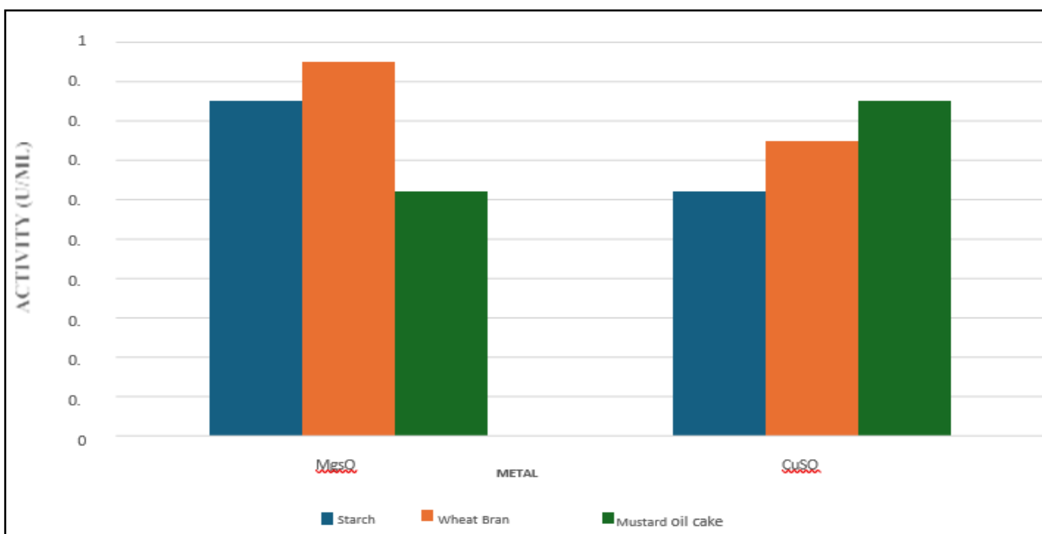

Fig 3: Effect of metal ion on production of amylase enzyme

4.4. Effect of time on enzyme production

Fig no.4 shows effect of incubation time on the activity of the amylase enzyme, measured in Activity (U/ml)**.** The study compares three different substrates: starch, wheat bran, and mustard oil cake. On Day 1, enzyme activity was relatively low across all substrates, with wheat bran showing

the highest activity (0.12 U/ml), followed by starch (0.1 U/ml) and mustard oil cake (0.09 U/ml). By Day 2**,** amylase production increases, with wheat bran reaching the highest activity (0.2 U/ml), followed by mustard oil cake (0.15 U/ml), and starch (0.12 U/ml). O
Day 3**,** enzyme activity peaks across all substrates. Mustard oil cake exhibits the highest activity (0.28 U/ml), followed closely by starch (0.26 U/ml) and wheat bran (0.25 U/ml). On Day 4, the activity of enzyme decreased as for the mustard oil cake exhibits the lowest activity (0.16 U/ml), followed closely by starch (0.11 U/ml) and wheat bran (0.09 U/ml).

Overall, the data suggest that amylase activity increases over time, with maximum production observed after 3 days. Mustard oil cake and starch show better performance in the long run, whereas wheat bran initially supports higher activity but levels out later.
In other studies, it was reported that the incubation period varies with enzyme production. Short incubation period offers potential for inexpensive production of enzyme [Somjoy *et al.,* 1995] [11]. There reports were resulted that the mycelial growth on starch reached a maximum after 5 days and maximum amylase activity was produced after 2 days of cultivation [Ely *et al.,* 2002] [4]

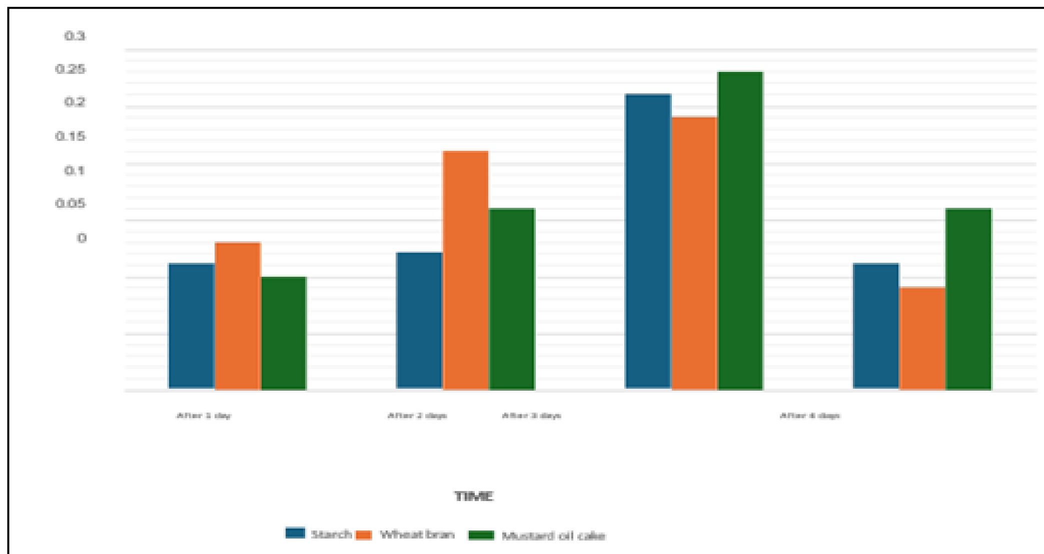

Fig 4: Effect of time course on activity of amylase

4.5. Effect of carbon sources
Fig no.5 shows effect of different carbon sources such as glucose, sucrose and lactose on the production of the amylase enzyme was studied. Among the three sources, sucrose leads to the highest enzyme production, with mustard oil cake showing the maximum activity (0.45 U/ml), followed by wheat bran (0.38 U/ml) and starch (0.35 U/ml). In contrast, glucose results in moderate enzyme activity, where mustard oil cake exhibits the highest value (0.35 U/ml), while wheat bran and starch remain lower (0.25 U/ml). Lactose supports intermediate amylase production, with wheat bran achieving the highest activity (0.35 U/ml), followed by mustard oil cake (0.3 U/ml) and starch (0.22 U/ml). These findings indicate that sucrose was the most effective carbon source for enhancing amylase production across all tested substrates. In other studies it was reported that glucose and sucrose supplementation resulted in the repression of enzyme production [Varalakshmi *et al.,* 2009] [14, 15].

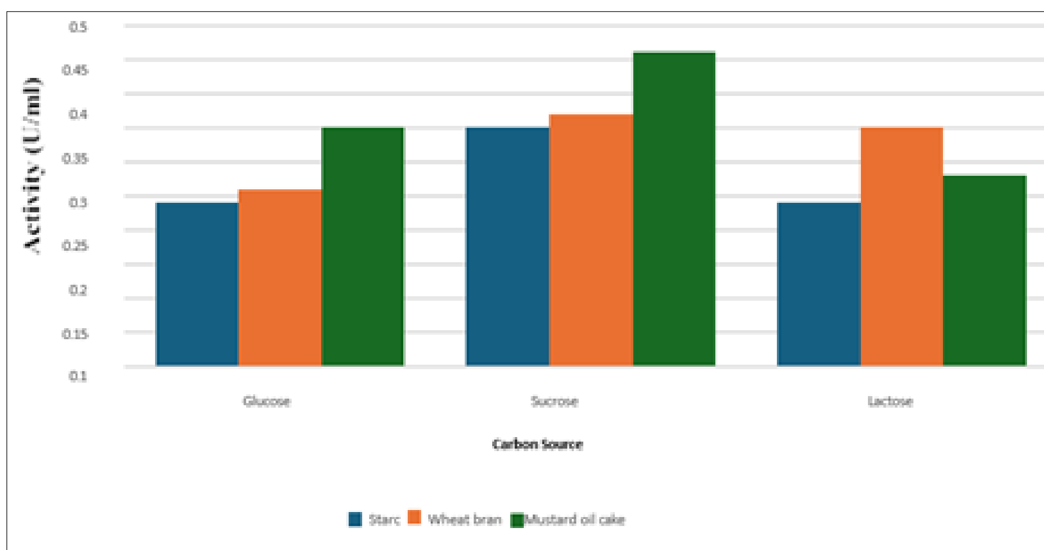

Fig 5: Effect of carbon source on production of amylase enzyme

4.6. Effect of nitrogen sources

Fig no.6 shows effect of different nitrogen sources on the production of the amylase enzyme using three substrates: starch, wheat bran, and mustard oil cake was studied. The nitrogen sources tested were ammonium sulphate, ammonium chloride, and sodium nitrate. Among these, ammonium sulphate resulted in the highest enzyme activity, particularly when mustard oil cake was used as a substrate, reaching approximately 0.45 U/ml. Ammonium chloride showed moderate enzyme activity, with mustard oil cake again yielding the highest production compared to starch and wheat bran. Sodium nitrate produced the lowest enzyme activity, with all three substrates showing nearly similar values. Overall, mustard oil cake proved to be the most effective substrate, especially when combined with ammonium sulphate, while sodium nitrate was the least effective nitrogen source for amylase production. Other results agree found that the nitrogen supplements enhance the production of the organwasm and have increased in the biomass cropped [Anupama and Ravindra, 2001] [1]. Previous findings have shown that peptone, sodium nitrate and casein hydrolysate are good nitrogen supplements for amylase production [Got *et al.,* 1998] [5].

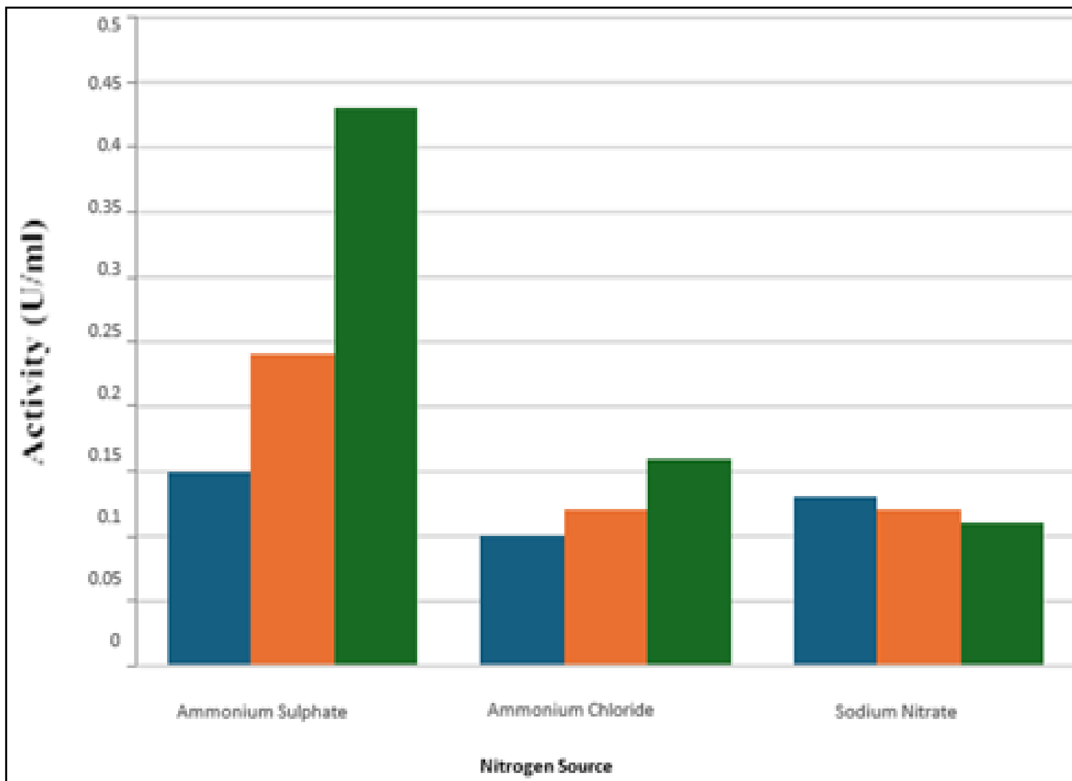

Fig 6: Effect of nitrogen source on production of amylase enzyme

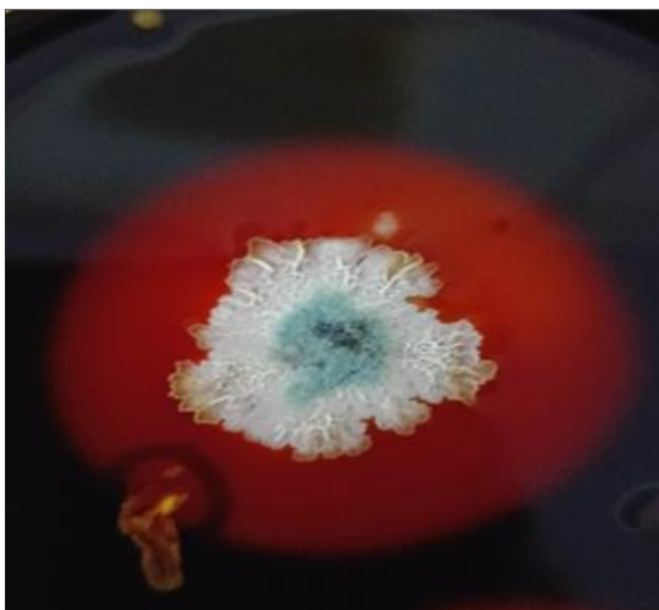

Fig 7: Screening of amylase producing *Aspergillus niger*

Fig 8: Production of amylase usin

Sr. No	Isolate	Amylase Producer
1.	A1	++
2.	A2	+++
3.	A3	+
4.	A4	+
5.	A5	++
6.	A6	+
7.	A7	+
8.	A8	++
9.	A9	+
10.	A10	+
11.	A11	++
12.	A12	+
13.	A13	+

Conclusion

Based on the above study it is concluded that Mustard oil cake can be a good substrate for the production of amylase and can be helpful in reducing the production cost. Other substrates like Wheat bran used in the study can also be used industrially for amylase production but after proper optimization. Optimizing fermentation conditions further enhanced production, making this approach cost-effective and environmentally sustainable. Utilizing agrowaste for amylase production not only reduces costs but also promotes waste valorization, with potential applications in food, textile, and biofuel industries

References

1. Anupama Ravindra P. Value-added food: Single cell protein. Biotechnology Advances,2001:*19*(7):527–559.
2. Balkan B, Ertan F. Production of α-amylase from *Penicillium chrysogenum* under solid-state fermentation by using some agricultural by-products. *Food* Technology and Biotechnology,2007:*45*(4):439–442.
3. Bhargav S, Panda B. P, Ali M, Javed S. Solid-state fermentation: An overview. Chemical and Biochemical Engineering Quarterly,2008:*22*(1):49–70.
4. Ely R, Supriyanto M, Rina S. Mycelial growth and amylase production by Aspergillus niger on starch-based media. Journal of Applied Microbiology,2002:*93*(4):687–693.
5. Got T, Caut L, Morin A. Effect of nitrogen sources on amylase production by Aspergillus niger. World Journal of Microbiology and Biotechnology,1998:*14*(5):661–666.
6. Hernandez MS, Rodríguez MR, Guerra NP, Rosés RP. Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries. Journal of Food Engineering,2006:*73*(1):93–100.
7. Kathiresan K, Manivannan S. α-Amylase production by *Aspergillus niger* under submerged and solid-state fermentation using agro-industrial wastes. Indian Journal of Biotechnology,2006:*5*(1):110–112.
8. Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. *et al* Advances in microbial amylases. Biotechnology and Applied Biochemistry,1999:*31*(2):135–152.
9. Ramachandran S, Patel AK, Nampoothiri KM, Francis F, Nagy V, Szakacs G, *et al*. Coconut oil cake—a potential raw material for the production of α-amylase. Bioresource Technology,2004:*93*(2):169–174.
10. Sajjad M, Choudhry R. Production of amylase enzyme by fungi using wheat bran as a substrate. *Pakistan Journal of Phytopathology,*2012:*24*(2):128–133.
11. Somjoy P, Nakamura R, Horikoshi K. Influence of incubation time on microbial amylase production. *Journal of Fermentation and Bioengineering,*1995:*80*(3):241–245.
12. Sun H, Zhao P, Ge X, Xia Y, Hao Z, Liu J, *et al*. Recent advances in microbial raw starch degrading enzymes. *Applied Biochemistry and Biotechnology,*2010:*160*(4):988–1003.
13. Ueno H, Kato M, Iwashit K. Temperature effect on amylase production by *Aspergillus niger*. *Applied Microbiology and Biotechnology,*1987:*26*(4):345–350.
14. Varalakshmi KN, Jaganmohan P, Kavitha M, Prabhakar T. Production and optimization of α-amylase from *Aspergillus niger*. Journal of Microbiology and Biotechnology Research,2009:3(2):55–60.
15. Varalakshmi KN, Jaganmohan P, Kavitha M, Prabhakar T. Production and optimization of α-amylase from *Aspergillus niger*. *Journal of Microbiology and Biotechnology Research,*2009:*3*(2):55–60