SOLID STATE FERMENTATION: A COST EFFECTIVE APPROACH FOR PRODUCTION OF STARCH LIQUEFYING FUNGAL AMYLASE USING AGRO INDUSTRIAL WASTES

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ABSTRACT: *Solid state fermentation (SSF) is one of the methods of interest for the production of cost effective products. Agro industrial wastes are reported as an appropriate substrate for the cost effective production of beta amylases. The aim of current study is the production of alpha amylase using agricultural wastes through SSF. Five indigenously isolated fungal strains including Aspergillus fumigatus KIBGE-IB33, Aspergillus flavus KIBGE-IB34, Aspergillus terreus KIBGE-IB35, Aspergillus niger KIBGE-IB36 and Aspergillus specie (Non-identified) were screened for beta amylase production using various agro industrial wastes. Among them, Aspergillus flavus KIBGE-IB34 produced maximum alpha-amylase at 30°C, pH 6.5 after 72 hours of incubation. SSF was carried out using different substrates namely corn cob, corn leaf, corn hairs, apple pulp, apple peel, banana peel, potato peel, wheat bran, rice husk and sugarcane bagasse. Rice husk (1.32 IU/ml) was found to be an ideal substrate for beta amylase production using SSF followed by banana peel (0.050 IU/ml)>potato peel (0.046 IU/ml)>corn hairs (0.037 IU/ml)>corn cob (0.029 IU/ml) and corn leaf (0.007 IU/ml). Utilization of agro-industrial wastes provides an alternative avenue and value-addition in cost effectiveness of commercial bioprocesses.*

Keywords: Beta amylase, *Aspergillus flavus*, Liquefaction, Rice husk

1. INTRODUCTION:

Amylase is an enzyme that is found in many forms. It can be divided into three categories; alpha, beta and gamma. All three types are glucoside hydrolases and take part in the process of starch degradation by acting on α-1,4-glycosidic bonds to create short-chain sugars. Amylase is a digestive enzyme that aids in the breakdown of carbohydrates by breaking the bonds between sugar molecules in polysaccharides. Many microorganisms such as bacteria and fungi secrete amylase in extracellular surrounding in order to carry out biochemical processes for their survival. Among these, fungi are considered as the most potent decomposer of organic materials in solid state fermentation than bacteria [1]. Some common fungi that have potential to produce amylase are *Aspergillus niger, Aspergillus candidus, A. ochraceus, Aspergillus flavus, Aspergillus terreus, Aspergillus fumigatus and others* [2]. For last few decades the use of starch and its derivatives in pharmaceutical and other industries has increased the use of amylases [3]. According to a study conducted in 2004, the global market for enzymes was approximately $2 billion and carbohydrases comprise 40% share of annual growth rate which is 3.3%. Among these carbohydrases, the annual sale of amylase is almost $11 million [4]. Several researches are being conducted in order to discover cost effective and efficient methods to minimize production cost of the amylase.

The aim of this study is the production of fungal amylase by using agricultural waste via Solid-State Fermentation which will make the cost more effective as amylases are widely used in food, textile, pharmaceutical and other industries. The main objective of this study is to find optimum parameters such as pH, temperature and fermentation period which are essential for the maximum yield of amylase.

2. MATERIALS AND METHODS:

2.1 Substrate:

Eleven agro-wastes containing starch as their essential component were used for amylase production in solid state fermentation (SSF). These include apple pulp, apple peel, potato peel, wheat bran, sugarcane bagasse, corn cob, corn stem, corn leaf, corn hairs and rice husk.

2.2 Microorganism:

Five fungal strains of *Aspergillus species* were selected and among them four were provided by The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE) whose 18S rRNA sequences has been deposited in GENBANK. These include *Aspergillus fumigatus* (IB-33), *Aspergillus flavus* (IB-34), *Aspergillus terreus* (IB-35) and *Aspergillus niger* (IB-36). One *Aspergillus* specie was locally isolated and was identified on the basis of its morphological characteristics. The culture was grown on plate containing Sabouraud dextrose agar (SDA), for 7 days at 30ºC and stored at 4ºC.

2.3 Inoculum preparation:

In order to prepare inoculum, fungal spores grown on 7 days old SDA medium were scraped gently and then aseptically transferred into 0.9% saline solution. The solution was vortex to obtain a homogenized mixture of fungal cells. Fungal cells were counted by Hemocytometer.

2.4 Screening of amylase producer:

The efficiency of fungal strains for producing amylase was screened in starch agar medium containing (g/l) yeast extract 5.0, peptone 5.0, starch 5.0, K_2HPO_4 1.0, $MgSO_4$ 0.5, Agar 22.0 and medium pH was kept 7.2. All the isolates were streaked on sterile solidified starch agar plates and all plates were incubated at 30ºC for 7 days. After 7 days all plates were flooded with iodine solution and zone of hydrolysis was observed.

2.5 Solid-state fermentation:

10g Agro waste substrate containing (g/100ml) 0.1g $MgSO_4$ and 0.1g K_2HPO_4 was taken in 250 ml flask and autoclaved at 121ºC for 20 minutes. Thereafter, the flask material was inoculated with 5 ml spore suspension of respective fungal strain and incubated at 30ºC for 7 days.

2.6 Optimization of enzyme production:

Besides darkness and moisture, there are certain other environmental factor which affect the ability of fungus for enzyme production. Therefore, different parameters were optimized for maximal enzyme production such as incubation temperature (25-40 ºC), pH (4.5-7.5) and fermentation period (24-168 h).

2.7 Recovery of enzyme:

For recovery of crude enzyme, 50 ml distilled water was added into the fermented mash and it was then placed on rotary shaker for 60 minutes. Thereafter, the mixture was filtered followed by centrifugation at 8000 rpm for 15 minutes. Thus, the cell free culture supernatant obtained was used as a source crude enzyme.

2.8 Enzyme assay:

The enzyme activity was assayed by adding 50µl enzyme into 50µl of starch buffer substrate (1%w/v) prepared in 0.1M phosphate buffer pH 7.0. The reaction mixture was incubated at 50ºC for 15 minutes. After incubation, 150µl of 3, 5-dinitrosalicylic acid reagent (DNS) was added in the mixture in order to stop the reaction followed by boiling for 15 minutes. Deionized water (750 µl) was added in the reaction mixture and was read against blank at 546 nm [5]. All the experiments were conducted in triplicates.

One enzyme activity unit was defined as "the amount of enzyme releasing 1 µmol of reducing sugar from the substrate in 1 minute at 50ºC".

3. RESULTS:

3.1 Verification of amylase producing strain:

After three days of incubation plate was flooded with Lugol's iodine solution and kept for 15 min at room temperature. Since amylase is produced extracellular, so it can be easily seen as zone of hydrolysis (Figure 1).

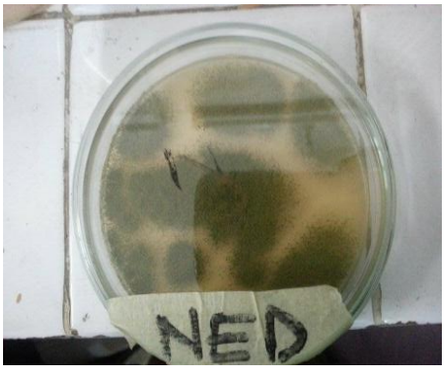

Figure 1: A. flavus culture

3.2 Suitable agro-waste:

Different flask containing different agro waste were inoculated with *Aspergillus* specie (Un-identified) and incubated at 30ºC for 7 days. After 7 days enzyme activity was measured. The highest enzyme activity was seen in flask containing rice husk (Figure 2).

3.3 Most potent fungal strain:

Different flask containing rice husk as a substrate were inoculated with five different fungal strains *Aspergillus niger*, *Aspergillus fumigates*, *Aspergillus flavus*, *Aspergillus terreus* and *Aspergillus* Strain (Non-identified). All flasks were incubated at 30 ºC for 7 days. After 7 days the maximum growth and enzymatic activity was found in *Aspergillus flavus* (0.2497 IU/ml) (Figure 3). Thus, it was selected for further studies.

3.4 Determination of optimum pH:

Enzyme production was found maximum (1.32±0.024 IU) when initial pH of the medium diluent was kept at 6.5. Further increase in the initial pH of the diluents resulted in decrease in enzyme activity (Figure 4).

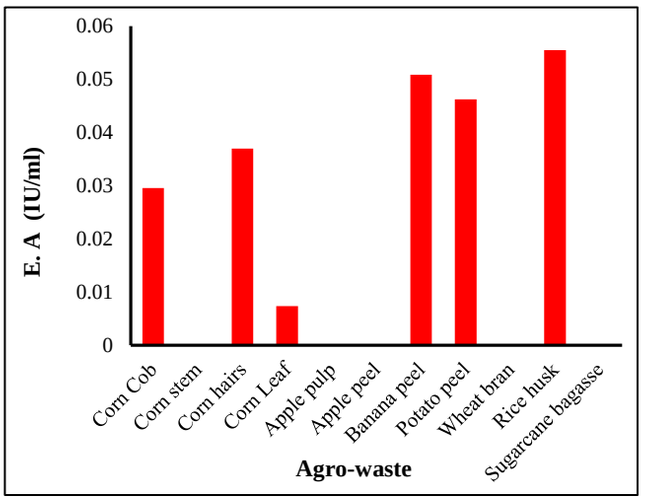

Figure 2: Effect of different Agra-waste on the production of amylase

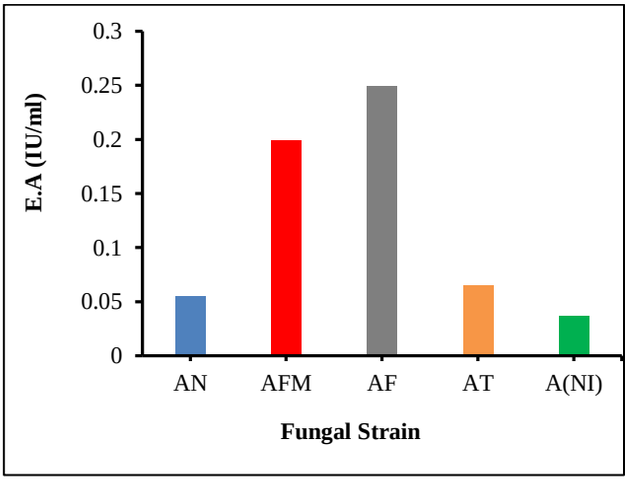

Figure 3: *Most potent fungal strain.*

AN: *Aspergillus niger*
AFM: *Aspergillus fumigatus*
AF: *Aspergillus flavus*
AT: *Aspergillus terreus*
A(NI): *Aspergillus* species, Non-identified

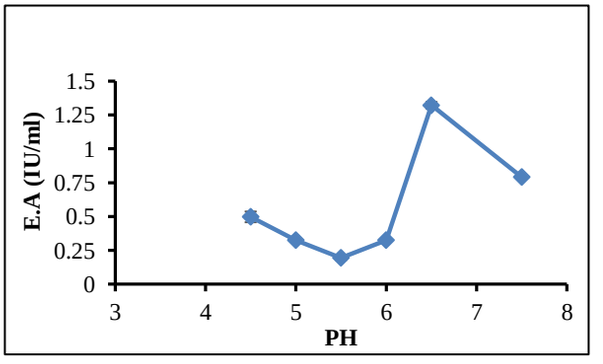

Figure 4: Optimized pH for fungal growth. Error Bars on graph shows SD of the enzyme activity among triplicates

3.5 Determination of optimum temperature for enzyme production:

The effect of temperature on fungal growth and amylase production from *A. flavus* strain was studied at different temperatures such as 25°C, 30°C, 35°C and 40°C. The optimum enzyme production was observed at 30°C (Figure 5)

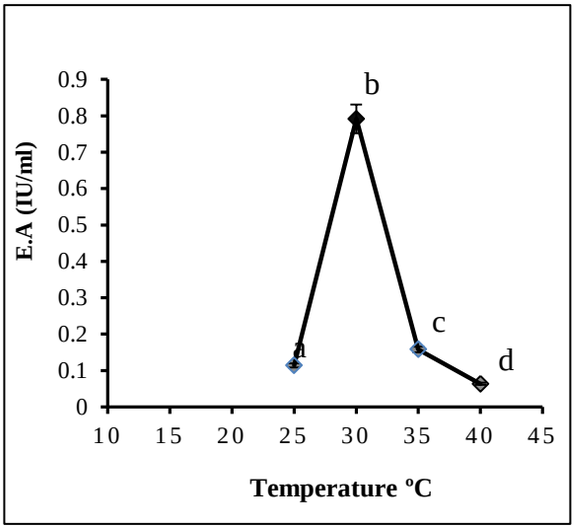

Figure 5: Optimized Temperature for fungal growth. Error Bars on graph shows SD of the enzyme activity among triplicates

3.6 Determination of optimum Incubation time:

All the pates were moistened with the media having pH 6.5, incubated at 30°C. Enzyme production was found maximum (0.75±0.02 IU) after 72 hours (Figure 6).

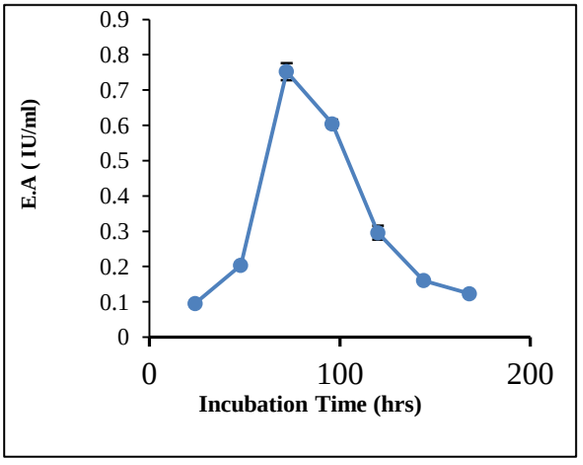

Figure 6: Optimized incubation period for fungal growth. Error Bars on graph shows SD of the enzyme activity among triplicates

4. DISCUSSION:

Different fermentable enviro-agro wastes which are dumped by food and beverages industries were used as a source for production of amylase which is commercially and widely used enzyme in different industrial processes. The present study showed that amylase production varied with the type of agro waste used. The maximum amylase production was achieved from rice husk (Figure 2). The study also showed that banana peel, potato peel, corn hairs, corn cob and corn leaf produced amylase 0.05 IU/ml, 0.046 IU/ml, 0.037 IU/ml, 0.03 IU/ml and 0.007 IU/ml respectively. Chimata *et al*., (2010) also reported rice husk as a good substrate for amylase production from *Aspergillus* specie [6]. Other investigators also reported that the production of amylase using rice bran and other rice components [7].

Figure 7: Growth of five different fungal strains using rice husk as substrate. From left to right, Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus and Aspergillus species

Once the agro-waste was selected the next step was to find out the highly potent fungal stain for amylase production. Results showed that *A. flavus* is the most potent fungal strain utilizing rice husk as substrate (Figure 3). It is worth to mention that other investigators also reported *A. flavus* as the most potent fungal strain for amylase production [8, 9,10].

The effect of different initial pH of the media (4.5-6.5) of the diluents on amylase production by *Aspergillus flavus* using solid state fermentation is shown in Figure 4. The fungal growth and enzyme production was maximally observed at pH 7.5. It was reported that initial pH of the medium has significant effect on extracellular production of amylase by microbes as it helps in carrying out different enzymatic reactions and transport of various substances across the microbial membrane [11]. It was also reported by other researcher that the maximum production of amylase was found at pH 6.0 from *A. flavus* [12]. Khan et.al, reported pH 6.2 as optimum for production of A.niger [12,13]. Some researchers also noted that maximum amylase production under SSF using *Aspergillus* specie was found at pH 6. [14] Our result is in accordance with P. Saranraj who reported pH optima for fungal amylase ranging from 2-12. [15]

Being a mesophilic fungus, the membrane of the *A. flavus* isolate was stable when the incubation temperature is in the mesophilic range and provided maximum conversion of the substrate to reducing sugars. It was reported that the enzyme production decreases when temperature other than optimum is provided, this is due to reduction in metabolic activity and impaired action of the cell membrane of the fungus [10]. It was found that maximum production of α-amylase was achieved at 30 °C as reported earlier by other workers in case of amylase production by *Penicillium fellutanum* and *A. flavus* [10]. Other investigators also mentioned that amylase production was maximum at an optimum temperature ranging from 25-37°C [16, 17].

It has already been suggested that fungal growth varies with fermentation time and it was found that further decrease or increase in fermentation period below or above 72 hours resulted in the low amylase production which might be due to denaturation of the enzyme which is associated with time (Figure 6). It also might be due to the fact that beyond this time period the production of by-products and toxic metabolites resulted in the

reduction of nutrients essential for the bacterial growth which play key role in enzyme production. It has also been reported that after 72 hours, *A. oryzae* showed maximum amylase production by utilizing rice bran as substrate [1]. F. S. Johnson et al., also reported maximum enzyme activity of fungal amylase after 72 hours of incubation. [18]

Thin layer chromatography (TLC) was performed and it was found that the spot of enzyme covers the same distance as covered by maltose (Figure 8) and R_f values of both maltose and the reaction mixture of enzyme and substrate is also same. It means that maltose is being produced by this enzyme as an end product and the production of maltose indicated the presence of Beta amylase enzyme. Some oligosaccharides were also found which showed that amylase enzyme also has debranching activity. It was also reported that *A. flavus* isolated from Ogun state of Nigeria also produces β-amylase [19].

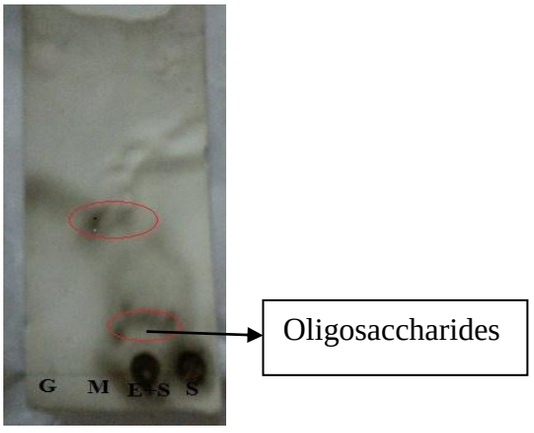

Figure 8: TLC with 100ul CFF. G=glucose, M=maltose, E=enzyme, S=starch

5. CONCLUSION:

It has been demonstrated that *Aspergillus flavus* IB-34 has the potential to produce β-amylase via fermentation of a cheap and inexpensive enviro-agricultural waste, rice husk. An interesting scope for future research includes optimization of various carbon and nitrogen sources for high yield of enzyme, purification and study of enzyme kinetics for industrial application of enzyme.

6. REFERENCES:

1. Silambarasan, S., Abraham, J. (2013) Comparative analysis on immobilized cells of *Aspergillus oryzae* and *Bacillus cereus* in production of amylase by solid state and submerged fermentation, European Journal of Experimental Biology, 3(3) 178-183.
2. Saleem, A., Ebrahim, M. K. H. (2014) Production of amylase by fungi isolated from legume seeds collected in Almadinah Almunawwarah, Saudi Arabia, Journal of Taibah University for Science, 8(2), 90–97.
3. Das, S., Singh, S., Sharma, V., Soni, M. L. (2011), Biotechnological applications of industrially important amylase enzyme. International Journal of Pharma and Bio Science, 2(1), pp. 486-496.
4. Sivaramakrishnan S., Gangadharan D., Nampoothiri K. M., Soccol C. R. & Pandey A. (2006) α-Amylases from Microbial Sources- An Over View on Recent Developments. Food Technology and Biotechnology 44(2) 173-184, 2007.
5. Gail Lorenz, M. (1959). Use of DinitrosaIicyIic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry, 3(3), 426–428.
6. *Chimata, N. K., Sasidhar, P., Challa, S., (*2010).Production of extracellular amylase from agricultural residues by a newly isolated Aspergillus species in solid state fermentation. African Journal of Biotechnology, 9(32) 5162-5169.
7. Kathiresan, K., Manivannan S. (2006) α-Amylase Production By *Penicillium fellutanum* isolated from mangrove rhizosphere soil, African Journal of Biotechnology, 5(10) 829-832.
8. Oyeleke, S. B., Egwim, E. C., Auta, S. H. (2010) Screening of *Aspergillus flavus* and *Aspergillus fumigatus* strains for extracellular protease enzyme production, Journal of Microbiology and Antimicrobials, 2(7): 83-87.
9. Sidkey N. M., Abo-Shadi M. A. A. R., Al-Mutrafy, A. M., Sefergy, F., Al-Reheily, N., (2010) Screening of microorganisms isolated from some enviro-agroindustrial wastes in Saudi Arabia for amylase production, Journal of American Science,6(10):926-939.
10. Bhattacharya S., Bhardwaj S., Das A., Anand S., (2011). Utilization of sugarcane bagasse for solid-state fermentation and characterization of α-amylase from Aspergillus flavus isolated from Muthupettai Mangrove, Tamil Nadu, India, Australian Journal of Basic and Applied Sciences, 5(12): 1012-1022
11. Ellaiah, P., Srinivasulu, B. Adinarayna, K., (2002) A review on microbial alkaline proteases. Journal of science and industrial research, 61: 690-704.
12. Singh H., Soni S. K., (2001) Production of starch-gel digesting amyloglucosidase by *Aspergillus oryzae* Hs-3 in solid state fermentation. Process Biochemistry, 37(5), 453–459.
13. Khan, J. A., & Yadav, S. K. (2011). Production of alpha amylases by Aspergillus niger using cheaper substrates employing solid state fermentation. International Journal of Plant, Animal and Environmental Sciencies, 1(3), 100–108.
14. Zambare, V. (2010). Solid State Fermentation of Aspergillus oryzae for Glucoamylase Production on Agro residues. International Journal of Life Science, 4, 16–25.
15. Saranraj, P., & Stella, D. (2013). Fungal Amylase - A Review, International Journal of Microbial Research, 4(2), 203–211.
16. Ramachandran S, Patel A.K., Nampoothiri K.M., Francis F., Nagy V., Szakacs G., Pandey A., (2004) Coconut oil cake- a potential raw material for the production of α-amylase, Bioresource Technology, 93(2):169-174
17. Jensen B., Olsen J. (1992) Physicochemical properties of a purified alpha-amylase from the thermophilic fungus *Thermomyces lanuginosus*, Enzyme Microb Technol, 14(2): 112-116
18. Johnson, F. S., Obeng, A. K., & Asirifi, I. (2014). Amylase production by fungi isolated from Cassava processing site. Journal of Microbiology and Biotechnology Research, 4(4), 23–30.
19. Oseni O. A., Ekperigin M. M. (2014) Activity of β-amylase in some fungi strains isolated from forest soil in south-western Nigeria, British Biotechnology Journal, 4(1)