Isolation and Identification of fungi producing amylase from locally made pap residue

Abstract

Amylase are enzymes that catalyze the hydrolysis of glycosidic bonds present in starch to release simple sugars and produce different monomeric products. Microbial amylase are essential enzymes compared with animals and plants amylases in industrial applications. Thus, in Eastern Nigeria, pap processing wastes (pap residues) from maize are generated in large amount during pap processing. This study was aimed to isolate, screen and identify potential fungi strains producing amylase from pap processing wastes (pap residue) and also utilize the pap residue for the production of reducing sugar. Fungi were isolated from the residue using standard microbiological procedures. Five (5) samples were collected from different places (Obikabia, Obete, Omoba, Eziama and Umuotuu) in Isiala Ngwa Abia state and were transported to the laboratory using a serial container for each. The samples were allowed to decomposed for 2 weeks. A 10-fold serial dilution were done by suspending 1 g of the sample in 9 ml of sterile distilled water. Thereafter, 0.1 ml dilution of each sample was plated on PDA plate and incubate at room temperature (25° c) for four days during which they were monitored and examined. The isolated fungi were tested for amylolytic activity using 1 ml of iodine solution and soluble starch containing PDA to screen for amylase production. Those that showed hydrolytic activity on the soluble agar were selected and identified microscopically by slide culture technique and lactophenol stain. The amylase activity was determined by submerged fermentation. Out of 21 fungal isolates obtained from sweet potato peel, 15 showed clear zone of hydrolysis. Out of the 15 fungal strains, some were the same fungi but different zone diameters as they were gotten from different places. Those that showed zone of hydrolysis with the corresponding number of fungal strains were; *Aspergillus fumigatus* (3) *Penicillin sp.* (2), *Mucor sp.* (1), *Trichophyton terrestre* (2), *Aspergillus niger* (1), *Aspergillus flavus* (3), *Microsporum canis* (1), *Sporothrix schenckii* (1), *White mould* (1), *Fusarium lichenicola* (1). Furthermore, the best five fungal isolates with the highest amylolytic potentials were selected for amylase production interms of glucose or reducing sugars released from the sweet potato peel during submerged fermentation. They include; *Aspergillus fumigatus*, *Sporothrix schenckii*, *Trichophyton terrestre* (E), *Aspergillus flavus, Trichophyton terrestre* (O). Among them, *Aspergillus flavus* had the highest glucose concentration at 8.387 mg\ml after 120 hrs of incubation. This result show that pap processing waste (residue) is a good source of amylolytic fungi and equally a good substrate for amylase production interms of glucose.

Keywords: Isolation, screening, fungi, Amylase, and pap residue

Introduction

A sizable portion of the global industrial enzyme markets are made up of amylase (E.C.3.2.1.1), which is used in various bio-industrial processes (Semira *et al*., 2021; Anbu and Hur, 2014). Enzymes are crucial in the food and energy industries because they catalyze the hydrolysis of starch into simple sugars, which are used as raw materials for many products (Da-Lagea *et al*., 2017; Prakash and Jaiswal, 2019; Hernandez *et al*., 2016). The glycosidase enzyme amylase, on the other hand, catalyzes the breakdown of the glycosidic bond in starch to produce smaller sugars useful to the bio industry. Microbial amylases come from bacteria, fungi, and yeast and are mostly used in industrial and scientific research (Santamaria.*et al*., 2019; Sing and Kumari, 2016). Industrial uses for amylases include textiles, pharmaceuticals, baking, paper, brewing, and food fermentation (Reddy *et al*., 2019; Sing and Kumari, 2016; Ezea *et al*., 2021). The advantage of obtaining amylases from microbes, particularly fungus, is that the organisms may be easily modified, and mass manufacturing of the enzymes is affordable (Ogbonna *et al*., 2014). Endoamylase and exoamylase are the two distinct subtypes of amylase. Endoamylase randomly catalyzes hydrolysis within the starch molecules. This results in the synthesis of oligosaccharides of different chain lengths, both linear and branched. The end products produced by the exoamylases, which hydrolyze the substrate from the non-reducing end, get shorter and shorter over time (Anbu, 2013; Suman and Ramesh, 2010; Samrat *et et.*, 2011; Rao *et al*., 2007; Parveen *et al*., 2011; Yakup *et al*.,

2010). The amylases are known as glycosidic bonds hydrolases because they break down α-1-4 glycosidic bonds.

The primary substrate for all alpha amylases (E.C.3.2.1.1) is starch, which they break down into small molecules of glucose and maltose (Adinarayana *et al*., 2015; Suman and Ramesh, 2010). The two glycose polymers amylose and amylopectin, which are composed of glucose molecules joined by glycosidic linkages, are the building blocks of starch. When internal α-1,4-glycosidic linkages in starch are hydrolyzed, products like glucose and maltose are produced. Alpha amylase is a hydrolase enzyme that catalyzes this process. Being a calcium metalloenzyme, its ability to function is dependent on the presence of a metal co-factor (Singh *et al.*, 2016; Puri *et al*., 2013; Mojsov, 2012; Silva *et al.*, 2011). Endo-hydrolase and exo-hydrolase are two different forms of hydrolase. Exo-hydrolase works on the terminal, non-reducing ends of the substrate molecules, whereas endo-hydrolase works anyplace on the interior of the substrate molecules. α-amylase have a greater tendency to act quickly than beta amylase because they can operate anywhere on the substrate. It is a significant digestive enzyme in mammals, and its optimal pH range is 6.7 to 7.0. (Elijah and Asamudo, 2016; Vitolo, 2019; Takahiro and Shigehiko, 2011; Sundarram and Krishna, 2014; Ugoh and Ijigbede, 2013).

Production of amylase depends on the strains, the cultivation or inoculation method, cell growth, nutrient requirements. Secondly, the cost of substrate for amylase production is a major problem, therefore choosing of an appropriate low cost effective substrate for the fermentation process will be a viable option.

Therefore, this study was aimed to isolates, identify fungi producing amylase from locally made pap residue and to evaluate its potentials ability in the production of amylase in form of glucose unit.

Materials and Methods

Sample Collection

Pap residue (Pap processing waste) sample was collected from different places in Abia State which includes; (Obikabia, Eziama, Obete, Omoba, Umuotuu). The sample was collected in a moist form and transported with a sterile container into the laboratory. It was placed under room temperature at 30° C for it to grow mould (decomposition) which stays for two weeks. One of the samples collect from another place (Amaku) was air dried under room temperature also for further analysis.

Preparation of Media

PDA preparation

Potato dextrose agar was prepared according to manufacturer's instruction. 15.6 g of PDA was weighed out and poured into a conical flask's. It was dissolved in 400 ml of water and homogenize using magnetic stirrer. After cooling an antibacterial drug chloramphenicol was added to it and mix properly. The media was sterilize using autoclave for 15 minutes at 121° C and allow to cool. Under a close flame it was dispensed into a sterile Petri dishes which was place inversely after solidification inorder to check for sterility test, for 24 hrs at room temperature.

Soluble starch media preparation

2.4 g of soluble starch was weighed out and 9.4 g of PDA was also weighed out, all where added together and dissolved in 240 ml of water. It was homogenized and sterilize by autoclaving for 15 minutes. It was allowed to cool and under a close flame it was poured into different sterile plates. After it most have solidified, the plates were placed inversely for 24 hrs inorder to check for sterility and were kept for further use.

Iodine stock preparation (indicator)

The iodine stock solution was prepared by dissolving 6 g of potassium iodide and 3g of iodine crystal in 100 ml of distilled water. The mixture was shaken properly until complete dissolution of the mixture. The volume was brought to 300 ml with distilled water. Then, it was stock in a reagent bottle for further use.

. **Isolation and Inoculation of fungi**

Serial dilution was done by using 6 test tubes and 9 ml of water in each of them. It was sterilized by autoclaving at 121° C for 15 minutes. The water was allowed to cool, before introducing the sample. The decomposed pap waste was transferred into the first test tube (10^{-1} ml) and mix well, using a pipette 1 ml from the initial test tube containing the sample was collected and transferred into the second test tube (10^{-2} ml), it was continued until 10^{-6} ml which at the end 1 ml was discarded so that they will have equal volume but different dilution of the sample. After serial dilution of all the 5 samples collected 0.1 ml was collected from (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) serial dilution and poured in each of the plate separately using spread plate technique. The L shaped glass rod was used to spread the sample around the plate under a close flame. (Remember the plate contains sterile solidified PDA media). It was then, incubated for 4 days at room temperature inversely.

Subculturing and Screening of fungi strains for amylase production

After the fungi has grown, it was subcultured inorder to get a pure culture by picking different fungi colonies from each plate and culture in different PDA plates under a close flame. It was place inversely for 4 days at room temperature.

Primary screening for fungi producers was done by using soluble starch and PDA plate method as started earlier. The fungi pure culture was inoculated into the sterile solidified media prepared. The plates were incubated inversely at room temperature for four days, for the growth and utilization of the starch by the organisms. After incubation, the plates were flooded with 1 % iodine stock solution for 5 minutes and was washed with Water to remove excess colour. Base on the highest size of zone of clearance around the well, the potential strains was selected from each plates and maintained on PDA slant using Bijou bottles (for the spores to remain alive). The zone of clearance was measured and recorded.

Identification of potential screened fungi strains

Those organisms that shows zone of clearance were identified using slide culture method. Aseptically with a pair of forceps, a sheet of filter paper was placed in a sterile Petri dishes. A sterile U-shaped glass rod was placed on top of the filter paper (note: rod can be sterilized by flaming, if held by forceps). Enough sterile water about 4 ml was poured on the filter paper to completely moisten it. With the help of a forceps, a sterile slide was placed on the U shaped rod. The scalped was sterilized by flaming gently and it was used to cut 5 mm square block of the medium from the PDA media prepared. The block of agar was picked up by inserting the scalped and carefully transfer the block aseptically to the center of the slide. The four side of the agar square was inoculated with fungi spores

or mycelial fragments of the fungus to be examined from the PDA slant. The cover slips were used to cover the agar in different plates and the inoculated organism. The Petri dishes cover was also used to cover the plate which was incubated for 7 days at room temperature.

The microscopic view of slide culture

The microscope was brought out and placed on the table, it was cleaned using lens tissue. A clean glass slide was placed in a tank and a drop of lactophenol was dropped on the center of the slide. With the help of forceps, the cover slip was picked from the cultured agar which has the spores and place on the slide containing lactophenol. The Iris diaphragm was closed and then, viewed under ×40 objective lens. The observation was taken and recorded at the results aspect.

Amylase production by fermentation process

To study the activity of amylase and enzyme production, submerged fermentation in a basal medium was done. The salts which were weighed includes $NaNO_3$ 2 g, $MgSO_4$ 0.5 g, KH_2PO_4 1g, KCL 0.5 g. and the dried locally made pap residue 10 g. They were all dissolved in 100 ml of distilled water in 250 conical flask and sterilized at 121^o C for 15 minutes. Thereafter cooling 10 ml of the different spores (2×10^7 spores/ml) were pipetted and added in different conical flask. The cultures were incubated in shakers incubator for 120 hours at 30^o C.

Determination of amylase in form of glucose

Glucose was determined according to Demoraes et al. (1999) using 0.5 ml of 0.5 % gelatinized soluble starch, buffered with 0.2 ml of 0.05 M citrate buffer (pH 4.8). The reaction mixture contained 0.2 ml citrate buffer, 0.5 ml of substrate solution (gelatinized soluble starch) and 0.3 ml enzyme solution. The reaction was carried out at 40^{o} C for 30 minutes. The reaction was stopped by addition of 1 ml of 3, 5 dinitrosalicylic acid reagents (DNSA) and boiled for 10 minutes followed by addition of 3 ml of distilled water. Thereafter, the absorbance was taken at 540 nm (Miller, 1959). One unit of amylase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar from soluble starch per ml per min under the assay conditions. A calibration curve was established with glucose from which reducing sugars were calculated.

Results

The total of 21 different fungal isolates were isolated at the initial stage. Out of the 21 fungal isolates isolated, 10 were positive on starch agar plate when screened and were identified as *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillin sp.*, *Mucor sp.*, *Trichophyton terrestre*, *Aspergillus niger*, *Sporothrix schenckii*, *Fusarium lichenicola*, *Microsporum canis* and *white mould.* Out of the 10 fungal isolates, five were selected based on the formation of clear zone of highest diameter on the starch agar as a result of hydrolysis caused by amylase production. They include; Obete 45 mm, Eziama 40 mm, Omoba 55 mm, Obikabia (AF) 35 mm, Obikabia (AD) 40 mm (table 1).

Table 2. shows the morphological appearance of isolated fungal in potato dextrose agar plate with the following characteristics; Obete (whitish fluffy sporulated surface), Eziama (white mould), Omoba (olive to dark green & white margin), Obikabia (AF) white, light

pink & dark brown to black at the center, Obikabia (AD) white, pink& brown at the center.

Table 1. Screening for amylase activity by the fungal isolates using starch agar

Source of fungal isolates	clear zone diameter mm (mm)
Obete	45
Eziama	40
Omoba	55
Obikabia (AF)	35
Obikabia (AD)	40

Table 2. Morphological appearance of the fungal isolates

Source of fungal isolates	Surface colour	Morphological description
Obete	whitish fluffy sporulated surface	colourless to dull yellow colour, flat growth, filamentous radiating irregular margin, raised, translucent.
Eziama	white mould	white & milky, fast, filamentous, smooth, convex, transparent.
Omoba	olive to dark green and white margin	colourless to dull yellow, fast growth, sporulated surface & irregular smooth edge, raised, transparent.
Obikabia (AF)	white, light pink and dark brown to black at the center	darkly pigment, slow at room temperature, filamentous, rough, umbonate, transparent.
Obikabia (AD)	white, pink and dark brown at the center	milky & brown, moderate, mycelial or filamentous, rough, umbonate, transparent.

Table 3. shows the microscopic characteristic or description of the fungal isolates in a slide culture plate and lactophenol stain. The fungal isolates were identified and compared with online fungi atlas as Obete (*Aspergillus fumigatus),* Eziama (*White mould),* Omoba (*Aspergillus flavus),* Obikabia (AF) (*Sporothrix schenckii),* Obikabia (AD) (*Trichophyton terrestre).*

Figure 1. shows the glucose concentration (reducing sugar) of the fungal isolates which was determined in submerged culture using spectrophotometer with DNSA reagent. From the graph it shows that *Aspergillus flavus* (8.387 mg/ml) and *Aspergillus fumigatus* (7.200 mg/ml) had the highest concentration of glucose after 96 to 120 hrs of incubation time.

Table 3. Identification of screened fungal isolates

Source of organisms fungal isolates	Microscopic morphology	Tentative fungi
Obete	Mycelium; septate branched hyphae, conidiophores, long erect, conidia	*Aspergillus fumigatus*
Eziama	Septate branched hyphae, rhizopus, regular, smooth shaped, conidiophore	*White mould*
Omoba	Mycelium: septate hyphae branched, conidiophore, conidia; globose to ellipsoidal.	*Aspergillus flavus*
Obikabia (AF)	Septate branched hyphae, conidiophore; hyaline in filamentous form, conidia.	*Sporothrix schenckii*
Obikabia (AD)	Septate hyphae branched multicellular filament, microconidia.	*Trichophyton terrestre*

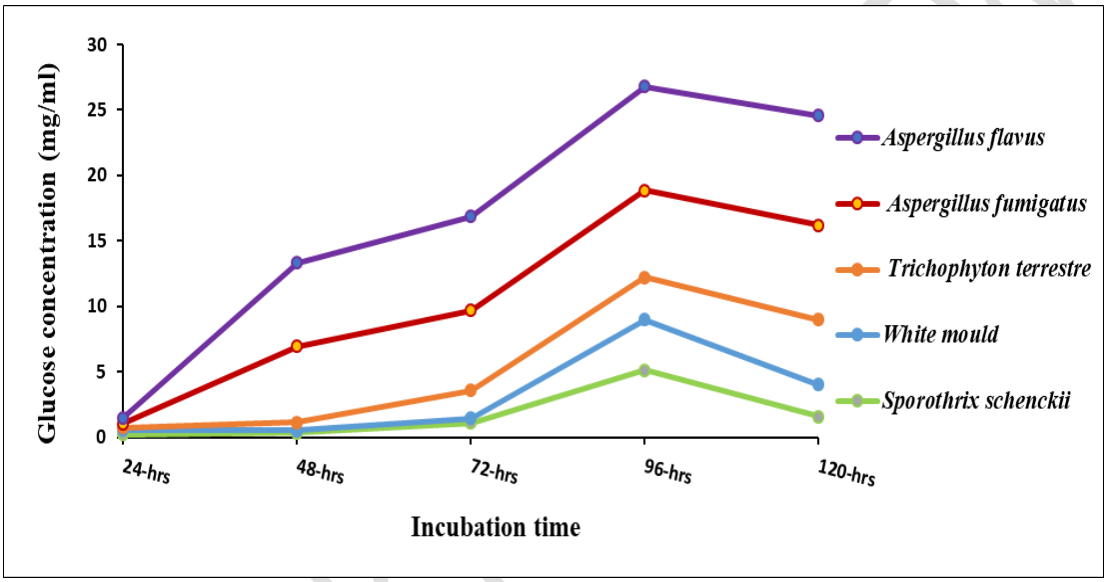

Fig. 1 Graph of time as against the glucose concentration of amylase activity for the fungal isolates.

Discussion

Microorganisms in particular have been regarded as treasures of useful enzymes. There is a great variation between various genera as to their ability to produce a specific enzyme, and the production of a particular enzymes varies with the particular medium depending on the organism involve in the reducing of sugar and its concentration. This study was focused on isolating and identifying fungi that produces amylase and to determine its glucose concentration. 21 fungi isolates were screened on starch agar plate which was flooded with iodine, 10 fungal isolates showed result and were identified microscopically as listed below with their zone of diameter clearance on starch agar plate which include; *Aspergillus flavus* 55 mm, *Aspergillus fumigatus* 45 mm, *white mould* 40 mm, *Sporothrix schenckii* 35 mm, *Trichophyton terrestre* 40 mm, *Penicillin sp*. 5 mm, *Mucor sp.* 5 mm, *Aspergillus niger* 20 mm, *Microsporum canis* 5 mm, *Fusarium lichenicola* 5 mm. Out of 10 fungal isolates, five are best producers of amylase and *Aspergillus flavus* has the highest zone of clearance. The same five fungal isolates were used to test for the concentration of reducing sugars (glucose) in a submerged fermentation medium. The results also showed that *Aspergillus flavus* (8.387 mg/ml) and *Aspergillus fumigatus* (7.200 mg/ml) has the highest concentration at 96 to 120 hrs of incubation time after plotting the graph with the wavelength of 540 nm, and are predominant in all the samples collected from different location. The results are in agreement with Ogbonna *et. al*., (2014) who reported that fungi isolated from soil and waste water sample obtained from garri processing site, during the screening of amylase producing fungal isolates shows that *Aspergillus flavus* has the highest zone of clearance (52 mm) which indicates more efficient amylase producing fungal isolate. In this paper, they studied isolation, screening and time course study of amylase producing fungi from garri processing environment.

This results are also in agreement with Gautam *et al*., (2013) who reported that the clear zone obtained when *A. flavus* (Afla-1) and *A. flavus* (Afla-2) were screened for amylase production was 84 mm and 82 mm, respectively on the average. They found out that *Aspergillus flavus* are best producing fungal isolates for amylase production and activity. Similar results were reported by Hussain *et al*., (2013) who observed 13.03 u/mg was the highest quantity of amylase produced by *A. flavus* at 96 hrs incubation period. Corbin *et al*., (2016) reported *Aspergillus flavus* as the best producing extracellular amylase among 136 fungal isolates screened for enzymes production revealed that dry weight, protein and amylase produced by the isolate was 7.22 mg/ml, 1059.08 µg/ml and 11.13 IU/ml. Elijah and Asamudo (2016); Negussie *et al*., (2019) stated that the medium used for amylase production monitored in relation to the incubation time (24-120 hrs) reflected a continuous increase in glucose concentration and amylase production. The trend is also in agreement with Raul *el al*., (2014) who monitored time course for amylase and glucose production during production and partial purification of alpha amylase from Bacillus subtilis using solid state fermentation.

Conclusion

Regarding the limitation of the current raw material use in glucose and amylase production with respect to cost, the following could be concluded: pap processing waste have the potential to be used as substrate in the production of glucose and amylase by fungal isolates from pap processing waste. Pap processing waste and its indigenous fungal isolates have a positive significant effect on the amylase interms of glucose production. However, Development of technologies for processing of pap processing waste into a useful metabolite such as glucose will guarantee market and prices and

reduce the cost of amylase and glucose. This study has demonstrated that fungal isolates from pap processing waste and pap processing waste itself, which is sufficiently found in the Eastern region of Nigeria and many tropical countries can be converted to amylase and glucose.

References

Adinarayana, K., Kugen, P., and Suren, S. (2015). Amylase production in solid- state fermentation by the thermophilic fungus *Thermomyces lanuginous*. *Journal of Biosciences and Bioengineering, 100(2):* 168-171.

Anbu, P. and Hur, B. K., (2014). "Isolation of an organic solvent-tolerant bacterium *Bacillus licheniformis* PAL05 that is able to secrete solvent-stable lipase," *Biotechnology and Applied Biochemistry, 61(5)*: 528–534.

Anbu, P., (2013). "Characterization of solvent stable extracellular protease from *Bacillus koreensis* (BK-P21A)," *International Journal of Biological Macromolecules, 56*: 162–168.

Corbin, J. M., Hashimoto, B. I., and Karuppanan K., (2016). "Semicontinuous bioreactor production of recombinant butyrylcholinesterase in transgenic rice cell suspension cultures," *Frontiers in Plant Science, 7*, article 412.

Da-Lagea, J.L., Etienne, G.J., Danchinc, E.G.J. and Casane, D. 2017. Where do animal α-amylases come from? An interkingdom trip. *Federation of European Biochemistry Society, 581*:3927–3935

Elijah, A.I. and Asamudo, N. U., (2016). Molecular characterization and potential of fungal species associated with cassava waste. *British Biotechnology Journal, 10 (4):* 1-15.

Ezea, I. B., Ezaka, E., Iwuagwu, J. O. and Ituboch, C. O. (2021). Biological production of citric acid in submerged culture of *Aspergillus niger* using cassava pulp wastes. *Archives of Ecotoxicology, 3* (3): 69- 74.

Gautam, N., Singh, K. P., and Modi, D. R., (2013). Screening of soil fungi for α-amylase activity. *Journal of Recent Advances in Applied Sciences, 28:* 110-112.

Hernandez, M.S., Rodriguez, M.R., Perez-Guerra, N. and Perez-Roses, R. (2016). Amylase Production by *Aspergillus niger* in Submerged Cultivation on two Wastes from Food Industries. *Journal of Food Engineering. 73*:93-100.

Hussain, I., Siddique, F., Mahmood, M. S., and Ahmed, S. I., (2013). " A review of the microbiological aspect of α-amylase production," *International Journal of Agriculture and Biology, 15(5):* 1029–1034.

1599 - 1616.

Mojsov, K., (2012). "Microbial α-amylases and their industrial applications: a review," *International Journal of Management, IT and Engineering, 2:* 583–609.

Negussie, F., Bussa, Mesfin, M., Manikandan, M., and Melese A., (2019). Isolation and characterization of amylase enzyme from selected fungal strains of wash Forest of North Shoa, Ethiopia. *Science Journal of Biology & Life Sciences. 1 (2):* SJBLS.MS.ID.000506.

Ogbonna, C. N., Okpokwu, N. M., Okafor, C. U., and Onyia, C. E., (2014). Isolation and screening of amylase producing fungi obtained from garri processing site. *International Journal of Biotechnology and Food Science, 2(5*): 88-93.

Parveen, J., Zulkarnain, I. M., and Alam, M. Z., (2011). Effects of physicochemical

parameters on the production of phenolic acids from palm oil mill

effluent under liquid-state fermentation by *Aspergillus niger* IBS-103ZA.

Food Chemistry, 124: 1595-S1602.

Prakash, O. and Jaiswal, N. (2019). Alpha-Amylase: An Ideal Representative of Thermostable Enzymes. *Applied Biochemistry and Biotechnology. 160*(8):2401-2414.

Puri, S., Arora M, and Sarao, L., (2013). Production and optimization of amylase and glucoamylase using *Aspergillus oryzae* under solid state fermentation. *Int. J. Res. Pure Appl. Microbiol*. 3(3): 83-88.

Raul, D., Biswas, T., Mukhopadhyay, S., Kumar Das, S., and Gupta, S., (2014). "Production and partial purification of alpha amylase from *Bacillus subtilis* (MTCC 121). Using Solid State Fermentation. *Biochemistry Research International*: 2014.

Sing, P. and Kumari, P., (2016). Isolation and characterization of amylase producing Bacillus spp. from selected soil sample. *Int. J. Res. Biosci*., *5(2)*: 24-29.

Singh, R., Mittal, A., Kumar, M., and Mehta, P. K., (2016). Amylases: A note on current applications. *International Research Journal of Biological Sciences, 5(11)*: 27-32.

Silva, T. M., Alarcon, R. F., Damasio, A. R. D., Michelin, M., Maller, A., Masui, D. C., Terenzi, H. F., Jorge, J. A., and Polizeli, M. D. T. M., (2011). Use of cassava peel as carbon source for production of amylolytic enzymes by *Aspergillus niveus*. *International Journal of Food Engineering, 5(5)*: 1-11.

Sundarram, A., and Krishna Murthy, T. P., (2014). "α-amylase production and applications: a review," *Journal of Applied and Environmental Microbiology, 2(4):* 166–175.

Ugoh S. C., and Ijigbade B., (2013). Production and characterization of amylase by fungi isolated from soil samples at Gwagwalada, FCT, Abuja–Nigeria. *Rep. Opin. 5(7):* 44-53.

Rao, D. M., Swamy, A. V. N., Siva Rhama and Krishna, G., (2007). Bioprocess technology strategies, production, and purification of amylases: An overview. *The Internet Journal of Genomics and Proteomics, 2(2)*: 30-34.

Reddy, R., Reddy, G. and Seenayya, G. (2019). Enhanced Production of Thermostable α-amylase of Pullulunase in the Presence of Surfactants by *Clostridium thermosulfurogenes* SV2. *Process Biochemistry, 34*: 87-92.

Samrat, C., Abhijeet, K., Ren, B., Wang, J., Xiang-Yang, L., and Kakasaheb, M., (2011). Characterization and stability studies on surfactant, detergent, and oxidant stable α-amylases from marine halo-alkaliphilic Saccharopolyspora sp. A9. *Journal of Molecular Catalysis B: Enzymatic, 68(1):* 52-58.

Santamaria, R.I.G., Delrio, G., Rodriguez, M.E., Soberan, X. and Lpezmumguia, A. (2019). Alcoholysis Reactions from Starch with Alpha Amylases. *FEBS Letter, 45*: 346 -350.

Semira, N. Y., Tamene, M. J. and Meera, I. (2021). Screening and Characterization of Thermostable Amylase-Producing Bacteria Isolated from Soil Samples of Afdera, Afar Region, and Molecular Detection of Amylase-Coding Gene. *International Journal of Microbiology, 10*: 9-10.

Suman, S. and Ramesh K., (2010). Production of a thermostable extracellular amylase from thermophilic Bacillus species. J. *Pharm. Sci. Res*.,2(2): 149-154.

Takahiro, K., and Shigehiko, H., (2011). Local entropy difference upon a substrate -binding of a psychrophilic α-amylase and a mesophilic homolog. *Chemical Physics Letters*, *501:* 517-522.

Vitolo M, (2019). Extraction of invertase from *saccharomyces cerevisiae*: effect of rheological parameters. *World Journal of Pharmaceutical Research*, *8(6):* 12-19.

Yakup, K., Ahmet, C., Ozlem, F., and Ali, O. B., (2010). Cloning, expression, and characterization of highly thermos and pH stable maltogenic amylases from a thermophilic bacterium *Geobacillus caldoxylosilyticus* TK4. *Process Biochemistry*, *4(6):* 821-828.