JOURNAL OF VETERINARY AND APPLIED SCIENCES
VOLUME 15, ISSUE 1: 885 – 892 (2025)
Published by: Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria
ISSN: 2315-6856; e-ISSN: 2636-5553; Website: www.jvasonline.com

Effects of pH on the activity of glucoamylase obtained from *Aspergillus niger* in a submerged fermentation process using guinea corn starch as carbon source

Gideon E. Onunwa [1] *, Arinze L. Ezugwu [1, 2], Ferdinand Chilaka [1], Solomon C. David [3], Nmesoma M. Ike [1], Precious Charles [1], Amarachi G. Nwachukwu [4]

[1] Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

[2] Department of Genetics and Biotechnology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

[3] Department of Veterinary Physiology and Pharmacology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria.

[4] Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

==========

Abstract

Amylase enzymes play a crucial role in starch processing industries as they break down polysaccharides to simple sugars. Amylases are produced by microbial organisms for industrial use. The use of microorganisms, especially fungal species, and the employment of low cost substrates such as agricultural products for amylase production serves as an economical means for obtaining the enzymes. Glucoamylase (C3009H4570N782O1012S13) is an exoenzyme that removes glucose units sequentially from the non-reducing ends of starch and oligosaccharides. Glucoamylase also cleaves the α-1, 6- and α-1, 3-bonds, although at a slower rate. Glucoamylase is used in the processed-food industry, fermentation technology, textile and paper industries. In the present study, *Aspergillus niger* was isolated and sub-cultured for the production of glucoamylase, using submerged fermentation process. An eleven-day experimental study was carried out to determine the day of peak glucoamylase activity and protein production. Glucoamylase activity was assayed using Bernfeld's or 3, 5- dinitrosalicyclic acid (DNSA) method, while protein was determined using the Lowry's method. Peak glucoamylase activity was obtained on the 6th day of the submerged fermentation using guinea corn starch as a carbon source. The crude glucoamylase harvested on day six had an enzyme activity of 208.5459 µmol/min with a total protein estimation of 214.95 mg in 150 ml. The crude enzyme was subjected to ammonium sulphate precipitation using 70% ammonium sulphate saturation to precipitate protein with the highest glucoamylase activity, and gel filtration chromatography was then used to further purify the precipitate obtained from the ammonium sulphate precipitation, which gave activities of 125.82 µmol/min and 83.09038 µmol/min, respectively. The optimum pH for glucoamylase activity was 5.0. The high glucoamylase activity and optimum pH presents this enzyme as a potential alternative for industrial application.

Keywords: *Aspergillus niger*; Activity; Glucoamylase; pH; Specific activity.

*** Correspondence:** Gideon E. Onunwa; Email: ebubechi.onunwa.235572@unn.edu.ng; Phone: +2348184391147

Article History: Initial manuscript submission received – May 29, 2024; Final revised form received – February 25, 2025; Accepted for publication – March 03, 2025; Published – March 12, 2025.

Introduction

The acidity or alkalinity of a solution where a reaction is taking place depends on the concentration of the hydronium ion (H_3O^+) and the hydroxyl (OH^-) ions present in the solution. Mathematically pH is defined as the decimal logarithm of the reciprocal of the hydrogen ion activity in the medium (Karastogianni *et al*., 2016): $pH = - \log_{10} H^+ = \log_{10} (1/H^+)$. pH measurement is important for application in many areas of biochemistry, medicine, biology, chemistry, agriculture, engineering, food science and nutrition. pH is also very important in living systems through its role in regulating biochemical reactions (Karastogianni *et al*., 2016).

Enzyme activity is affected by the pH of the solution they react in; at very acidic and alkaline pH the structure of the enzyme catalytic site is altered causing it to no longer be a complementary site to its specific substrate, thereby inhibiting the reaction (Okamoto *et al*., 2017). The study of pH is very important in determining the optimum pH conditions of an enzyme and hence its stability (Okamoto *et al*., 2017). EC 3.2.1.3, glucoamylase, exo-1,4-α-D-glucan glucohydrolase, is an extracellular-amylase that functions by hydrolyzing α-1,4 and, to a lesser degree, α-1,6 glucosidic linkages, thereby liberating β-D-glucose from the starch's non-reducing ends (Karim and Tasnim, 2018). Glucoamylase is also able to completely hydrolyse starch when starch is incubated for long period of time and is hence known as the saccharifying enzyme (Karim and Tasnim, 2018). These multi-domain enzyme have an O-glycosylated linker region connecting the starch-binding domain to the catalytic domain (Marin-Navarro and Polaina, 2011). Only the catalytic domain is needed for complete dextrin hydrolysis (Lee and Paetzel, 2011), and the starch binding domain plays a role of binding and digestion of raw starch (Li *et al*., 2013). In the food industry, glucose amylase is crucial to the processing of starch. For instance, it helps to make fructose and glucose syrup from liquefied starch. Above all, it is utilized in the synthesis of glucose, which is a substrate for biological fermentations that produce ethanol. Additionally, it makes barley mash better for making beer (Adefisoye and Sakariyau, 2018).

Numerous microorganisms, such as bacteria, yeast and fungi, are able to produce glucoamylase (Kumar *et al*., 2012). Filamentous fungi constitute the primary sources for glucoamylase especially *Aspergillus niger* (Karim and Tasnim, 2018). Conventionally glucoamylase is produced by submerged fermentation (SmF) using fungi (Radha *et al*., 2012), however, the use of solid-state fermentation (SSF) methods to produce this enzyme has grown in popularity recently (Deshmukh *et al* 2011).

Glucoamylase has a wide range of uses in the food processing sector, bioethanol, confectionery, paper manufacturing, fabric industries, bakery products, and pharmaceuticals (Pathak and Sandhu, 2019). The present study determined the day of maximum release of glucoamylase obtained from *A. niger* using starch from guinea corn as source of carbon in a submerged fermentation system and evaluated how pH affects the activity of the glucoamylase harvested.

Materials and Methods

Chemicals and Reagents: Sephadex G-100 and bovine serum albumin (BSA), used for the study, were acquired from Sigma Chemical Company Limited (USA). Also, Folin–Denis reagent was bought from Sigma-Aldrich in the United States. Purchases of Tris HCl salt and ammonium sulphate were made from the British Drug House (BDH) Chemicals Limited (USA). The remaining compounds utilized during the research project were all of analytical grade and came from authorised vendors.

Collection of Plant Material: The guinea corn seeds used for the study were sourced from Ogige main market in Nsukka, Enugu State, Nigeria. Viability tests on the seeds were conducted at the Department of Botany, University of Nigeria, Nsukka, under supervision.

Processing of Guinea Corn Starch: The guinea corn starch was processed based on the method described by Agboola *et al*. (1990). After being sun-dried, the seeds were milled into fine flour. Three hundred gramme of flour was suspended in three litres of distilled water for a duration of 24 hours. Cheesecloth was used to filter the flour that was suspended. At room temperature, the starch extracted was left to settle for four hours. After removing the supernatant through decantation, the starch was twice rinsed with three litres of distilled water and then left standing for four hours. Afterward, the liquid lying above the solid residue was poured off. The moist starch obtained was left in the open to dry. The dried starch powder was kept at room temperature after being sealed in an airtight container.

Isolation of Glucoamylase Producing Fungi: The fungi that produce glucoamylase was isolated by using the procedure outlined by Martin *et al*. (2004). Identification of the microorganism was carried out using the method of Barnett and Hunter (1972).

The Fermentation Broth for Enzyme Production: The submerged fermentation (SmF) approach was used for the enzyme production; it made use of a flask of Erlenmeyer with 2.2 litre of sterile growth culture tailored for glucoamylase containing 6.6 g $(NH_4)_2SO_3$, 13.2 g NaH_2PO_4, 2.2 g $MgSO_4.7H_2O$, 0.22 g $FeSO_4$ and 18.2 g guinea corn starch. From the 2.2 litre of the sterile growth culture tailored for the production of glucoamylase, 100 ml was filled into each twenty-two 250 ml conical flask, marked day zero to day ten, making up two sets. To guarantee sterility, these flasks were sealed with aluminum foil and autoclaved for 15 minutes at 121 °C. Fresh plates were made as earlier defined and inoculated from the PDA slants. The cultures used to inoculate the flasks were three days old. Using a 10 mm cork borer, two discs with the corresponding isolates of fungi were introduced to each sterile flask, which were then securely closed. Incubation of the growth medium lasted for eleven days at room temperature. A flask was chosen and the mycelia biomass was separated by filtering for every day of harvest. Up until the eleventh day of fermentation, the filtrate's extracellular protein content and glucoamylase activity were measured daily.

Enzyme Mass Production: Following eleven days of submerged fermentation pilot tests, the maximum day of activity of glucoamylase was selected for the enzyme's mass/flow production. The procedure outlined above was utilized to produce two litres of the enzyme utilizing multiple Erlenmeyer flasks. On the day of maximal glucoamylase activity, enzyme harvesting was done. The medium was charged with 6.0 g $(NH_4)_2SO_3$, 12 g NaH_2PO_4, 2.0 g $MgSO_4.7H_2O$, 0.2 g $FeSO_4$ and 17.165 g starch of guinea corn, for glucoamylase production. A total of 2000 ml of the enzyme, was produced.

Protein Determination: Using the method outlined by Lowry et al. (1951), the amount of protein in the enzyme was determined, using bovine serum albumin as standard.

Glucoamylase Assay: The glucoamylase activity was determined using the method outlined by Bernfeld, (1951). The optical density of the colour generated was measured using a Surgicare UV/VIS spectrophotometer. Under the assay factors, one unit of the activity of glucoamylase is defined as the quantity of enzyme required for releasing one micro mole (µ mol) of glucose for each minute. A glucose standard curve was used to determine the released glucose concentration.

Purification of Enzyme: The purification of the raw enzyme was done using ammonium sulphate precipitation and size exclusion chromatography. It was discovered that 70% ammonium sulphate saturation was appropriate for the crude enzyme to precipitate the enzyme that was extracted on day six. This was maintained for 30 hours at 4°C, and after 10 minutes of centrifugation at 3500 revolutions per minute, the precipitates were then dissolved again in 40 ml of 20 mM acetic acid buffer of pH 5.0, and the precipitate was stored refrigerated for further use. The protein and glucoamylase activities were measured as previously mentioned. Five millilitre volume of the precipitate of the enzyme was added into a size exclusion chromatographic column and size exclusion chromatography employing sephadex G-100, which had been previously conditioned with a pH 5.0, 50 mM sodium acetate buffer, was conducted on it. A Surgicare UV/VIS spectrophotometer set to 750 nm was utilized to measure the concentration of protein for each fraction at 750 nm in accordance with the protocol described by Bernfeld, (1951). Each fraction's glucoamylase activity was measured as previously mentioned, utilizing the active fractions combined, and kept at -10˚C for storage.

Characterization of Enzyme: The effect of the pH on the partly purified glucoamylase was characterized. The ideal pH for the activity of glucoamylase (the enzyme generated on the sixth day of the submerged fermentation process) was ascertained by varying the pH by 0.5 in three different buffers: 20 mM sodium acetate pH 3.0 - 5.5, 0.1 M phosphate buffer pH 6.0 - 7.5, and 20 mM Tris-HCl buffer pH 8.0 - 10.0. 0.5 ml of each of the enzyme was incubated for 20 minutes in 20 mM of each buffer together with 0.5 ml of 1% guinea corn starch. The glucoamylase activity was observed at these different pH intervals in the same manner as previously stated.

Results

Experimental studies: The glucoamylase activity for the eleven (11) days of the pilot study is shown in Figure 1. The maximum concentration of protein was acquired during the fifth day, with the least concentration of protein acquired during the tenth day. On the sixth day, the highest glucoamylase activity was recorded (202.8061 µmol/min); while day zero recorded the least activity (5.8309 µmol/min) with specific activity of 70.2266 U/mg protein and 3.7151 U/mg protein respectively.

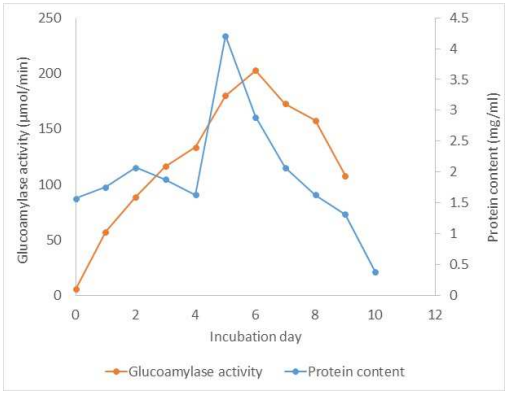

Figure 1: Glucoamylase activity and protein concentration at various days of incubation (day 0 to day 11) using guinea corn starch as substrate. Highest glucoamylase activity was obtained on day six, while the highest protein concentration was obtained on day five.

Glucoamylase Purification: An overview of the changes corresponding to every stage of purification carried out on the glucoamylase obtained from *Aspergillus niger* harvested on day six using submerged fermentation process is given within Table 1. Initially, the crude extract had a total protein content of 1818.8 mg and a total activity of 208,545.9 U, with a specific activity of 114.66 U/mg. After ammonium sulfate precipitation, there was a significant reduction in both total protein and activity; however, gel filtration improved the enzyme's purity, achieving the highest specific activity (145.11 U/mg) and a purification fold

of 60.33. The overall percentage yield after gel filtration was 39.84%, indicating substantial purification (Table 1).

The precipitation of glucoamylase using ammonium sulfate at different saturation levels is presented in Figure 2. The results indicate that enzyme activity was predominantly retained in the pellet fraction, with a peak activity observed at 70% saturation, followed by a steep decline beyond this point. Meanwhile, the supernatant fraction showed a corresponding decrease in enzyme activity, suggesting that the enzyme was effectively precipitated at this concentration.

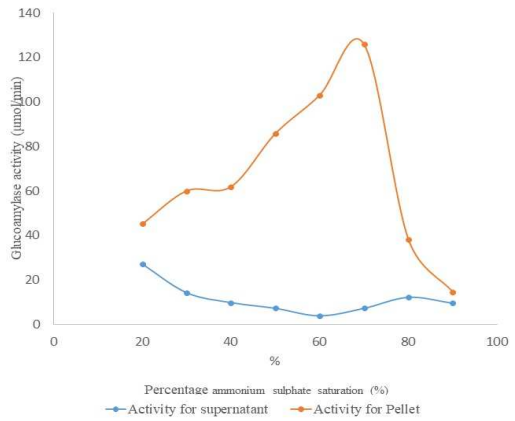

Figure 2: Ammonium sulphate precipitation profile for *Aspergillus niger* glucoamylase. Seventy percent (70%) ammonium sulphate saturation was found suitable to precipitate protein with highest glucoamylase activity (125.82 µmol/ min).

The elution profile of glucoamylase after gel filtration chromatography is presented in Figure 3. The enzyme exhibited a sharp peak in absorbance at 540 nm around tube numbers 10–12, indicating the elution of highly concentrated glucoamylase fractions. This sharp increase suggests that the enzyme was effectively separated during this stage of purification, coinciding with the fraction where the highest protein concentration and activity were recorded. Following this peak, the absorbance gradually declined across the subsequent tube numbers, indicating the separation of less concentrated enzyme fractions.

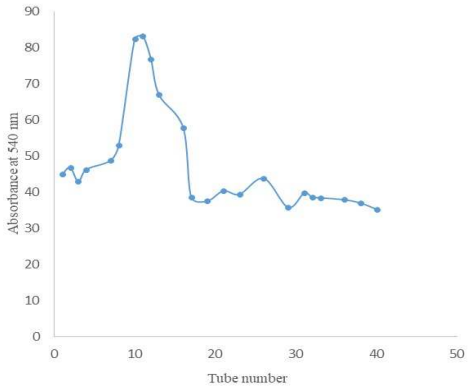

Figure 3: Elution profile of glucoamylase (harvested on day six of submerged fermentation) during gel filtration on sephadex G 100. A volume of 5 ml of 50 fractions were collected at an elution rate of 5ml per 20 min. Glucoamylase activity was assayed in each of the fractions collected.

Characterization of glucoamylase – *Optimum pH:* Figure 4 shows the effect that changes in pH has on the activity of glucoamylase (using guinea corn starch as substrate). An increase in enzyme activity was observed as the pH was increased from 3.0 to 5.0. Beyond this pH, the enzyme activity declined.

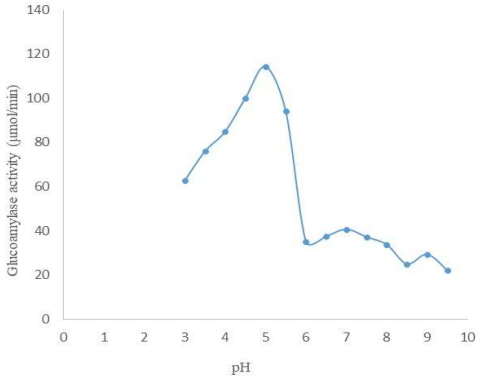

Figure 4: Effect of pH on glucoamylase activity harvested on day six of submerged fermentation. Optimum pH was obtained at pH 5.0 using guinea corn starch as substrates.

Table 1: Purification table for glucoamylase from *Aspergilus niger* harvested after six days of fermentation using guinea corn starch as carbon source.

	Crude	$(NH_4)_2SO_4$	Gel filtration
Volume (ml)	1000	250	60
Protein (mg/ml)	1.8188	1.2192	0.151
Total Protein (mg/ml)	1818.8	304.8	4.53
Activity (µmol/min)	208.5459	125.82	83.09038
Total activity (U)	208545.9	31455	4985.4
Specific activity (U/mg)	114.6612	103.1988	145.1106
Purification fold	1	0.015	60.33
Percentage yield	100	1.406	39.84

U (Unit) = µmole/min

Discussion

The findings of the present study which showed that the highest glucoamylase activity was on the sixth day of submerged fermentation process, is in agreement with the reports of Lawal *et al*. (2014). The decline in synthesis of glucoamylase starting on the seventh day to the eleventh day may be as a result of the presence of high glucose concentration obtained by the microorganism. Slivinski *et al*., (2011) reported in their study that the synthesis of glucoamylase by *A. niger* was reduced because of a high concentration of glucose in the organism, but when the concentration of glucose decreased in *A. niger* a further increase in the production of glucoamylase was observed as recorded in Figure 1.

The results obtained from the purification of the crude enzyme were similar to the results reported by Slivinski *et al*. (2011). According to their findings, glucoamylase precipitated at saturation levels of 60 – 85% ammonium sulfate saturation. Similarly, Mervat, (2012) reports 70% saturation of ammonium sulphate gave the maximum glucoamylase activity from JAN-25, a species of *Aspergillus* marine endophytes. Sephadex G 100 was used to carry out gel filtration. The activity of glucoamylase obtained on the sixth day declined from 208.5459 µmol/min to 125.82 µmol/min after precipitation using ammonium sulphate, and declined even more to 83.09038 µmol/min after gel filtration. This can be ascribed to the elimination of contaminants like other proteins, which raises the activity of the enzyme, and also the removal of cofactors or ions needed for the activity of the enzyme. This was similarly reported by Jebor *et al*., 2014 during the purification and characterization of glucoamylase obtained from *A. niger*. The gel elution profile showed two peaks in total: a major peak and a minor peak. This could suggest that *Aspergillus niger* synthesized two different isoforms of glucoamylase during fermentation (Nahid *et al*., 2012).

The results of the enzyme characterization show that the pH of 5.0 is the optimal pH with enzyme activity of 114.3404 µmol /min with starch of guinea corn as substrate. This aligns with the results reported by Ellaiah *et al.* (2002) and Deshmukh *et al* (2011), who reported that pH influenced the glucoamylase activity, with the maximum glucoamylase activity obtained at pH 5.0 for *A. niger* glucoamylase. Similar findings were reported by Nahar *et al*., (2008), who also reported that the ideal range of pH for the synthesis of glucoamylase was between 4.5 to 5.5. The

change in pH of the solution containing the enzyme can affect the enzyme active site by causing protonation or deprotonation of the side chain of amino acids present in the active site, resulting in loss of enzyme activity due to denaturation (Helms *et al.,* 1998).

Conclusion: Results of this study showed that day six gave the peak glucoamylase activity for glucoamylase produced by *Aspergillus niger* using submerged fermentation technique. The optimum pH of glucoamylase from *Aspergillus niger* was found to be pH 5.0. The activity of glucoamylase was noticed from the high acidic region of pH (3.0) before getting to its peak activity within the less acidic region of pH (5.0). This therefore, suggests that glucoamylase can function in acidic environments, making it important in industrial processes such as in high fructose corn syrup production, biofuel production and other fermentation biotechnological processes.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

The authors sincerely thank Prof. F. C. Chilaka of the Department of Biochemistry, University of Nigeria, Nsukka, for providing all the materials/resources used for this research.

References

Adefisoye SA and Sakariyau AO (2018). Production of glucoamylase by aspergillus niger in solid state fermentation. *Advances in Biological Research*, 12(1): 7 – 11.

Agboola SO, Akingbala JO and Oguntimi GB (1990). Processing of cassava starch for adhesive production. *Starch/Stakes*, 42: 12 – 15.

Barnett H and Hunter B (1972). Illustrated General of imperfect Fungi. 3rd edn. Burgess, Minneapolis, Minnesota, USA, pp. 20 – 130.

Bernfeld P (1951). Enzyme of starch degradation and synthesis. in: advances in enzymology. *Biochemistry*, 28: 61 – 68.

Deshmukh KB, Taur SA, Cherekar MN, Kothari MN and Pathak AP (2011). Process optimization, purification and characterization of glucoamylase from different Sorghum varieties. *Journal of Chemical and Pharmaceutical Research*, 3(2): 732 – 737.

Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P and Srinivasulu B (2002). Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated aspergillus species. *Process Biochemistry*, 38(4): 615 – 620.

Helms D, Helms C, Kosinski R and Cummings J (1998). Biology in the Laboratory, 3rd edn. New York, Freeman Publishing, pp. 9: 1 – 8.

Jebor MA, Ali ZM and Hassan BA (2014). Purification and characterization of the glucoamylase from *Aspergillus niger*. *International Journal of Current Microbiology and Applied Sciences*, 3(1): 63 – 75.

Karastogianni S, Girousi S and Sotiropoulos S (2016) pH: Principles and Measurement. In: Caballero B, Finglas P, and Toldrá F (Eds.). The Encyclopedia of Food and Health vol. 4, Academic Press, Oxford, pp. 333 – 338.

Karim MR and Tasnim T (2018). Fungal glucoamylase production and characterization: a review. *Bioresearch Communications*, 4(2): 591 – 592.

Kumar MS, Lakshmi MV, Sridevi V and Manasa M (2012). Glucoamylases: types, microbial sources and potential applications – a review. *International Journal of Science Innovations and Discoveries*, 2: 74 – 83.

Lawal AK Banjoko AM, Olatope SO, Alebiosu FA, Orji FA, Suberu YL, Itoandon EE, Shittu KA, Adelaja OD, Ojo E, Dike EN and Elemo GN (2014). Production and partial purification of glucoamylase from *Aspergillus niger* isolated from cassava peel soil in Nigeria. *African Journal of Biotechnology*, 13(21): 2154 – 2158.

Lee J and Paetzel M (2011). Structure of the catalytic domain of glucoamylase from *Aspergillus niger*. *Acta Crystallographica*, F67: 188 – 192.

Li Z, Wei P, Cheng H, He P, Wang Q and Jiang N (2013). Functional role of ß-domain in the *Thermoanerobacter tengcongensis* glucoamylase. *Applied Microbiology and Biotechnology*, 98(5): 2091 – 2099.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin Phenol Reagent. *Journal of Biological Chemistry*, 193(1): 256 – 275.

Marin-Navarro J and Polaina J (2011). Glucoamylase: structure and biotechnological aspects. *Applied Microbiology and Biotechnology*, 89: 1267 – 1273.

Martin N, de Souza SR, da Silva R and Gomes E (2004). Pectinase production of fungal strains in solid-state fermentation using agro industrial bioproduct. *Brazilian Archives of Biology and Technology*, 47: 813 – 819.

Mervat MA (2012). Production of glucoamylase by marine endophytic *Aspergillus* sp. JAN-25 under optimized solid-state fermentation conditions on agro residues. *Australian Journal of Basic and Applied Sciences*, 6: 41 – 54.

Nahar S, Hossain F, Feroza B and Halim MA (2008). Production of glucoamylase by Rhizopus species in liquid culture. *Pakistan Journal of Botany*, 40: 1693 – 1698.

Nahid P, Vossoughi M, Roostaazad R and Ahmadi M (2012). Production of glucoamylase by *Aspergillus niger* under solid state fermentation. *International Journal of Epidemiology*, 25(1): 1 – 7.

Okamoto AK, Miranda MA, Granjeiro JM and Aoyama H (2017). Enzymes with two optimum pH values. *World Journal of Research and Review*, 5(6): 5-11.

Pathak SS and Sandhu SS (2019). Screening of glucoamylase producing fungi from the soils of jabalpur region. *International Journal of Recent Scientific* Research, 10(1): 30470 – 30476.

Radha K, Srivastava AK, Ramaswamy NK, Suprasanna P and Souza SF (2012). Banana peel as substrate for alpha amylase production using *Aspergillus niger* NCIM 616 and process optimization. *Indian Journal of Biotechnology*, 11: 314 – 319.

Slivinski CT, Machado AVL, Iulek J, Ayub RA and Mendes de Almeida M (2011). Biochemical characterization of a glucoamylase from *Aspergillus niger* produced by solid state fermentation. *Brazilian Archive of Biology and Technology*, 54(3): 559 – 568.