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USE OF COMPOSITE AGRO-SUBSTRATES FOR AMYLOGLUCOSIDASE SYNTHESIS AND CHARACTERIZATION BY *Aspergillus niger* OTF AND *Aspergillus flavus* CLOR1 USING SOLID STATE FERMENTATION

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ABSTRACT

Amyloglucosidase catalyses the hydrolysisof glycosidic residues from non-reducing ends of amylose and amylopectin in starch components to produce β-D-glucose for industrial processes. The utilization of composite agro-substrates for amyloglucosidase production by*Aspergillus niger* OTF and *A. flavus* CLOR1 using solid state fermentation and characterization of the enzyme were investigated. The substrates include rice bran (R), soybeans flour (S), palm kernel cake (P), wheat bran (W) and oyster shell (O) at various compositions (Medium I – VIII) were supplied with adequate moisture content for a solid culture medium of fermentation.They were inoculated into with the isolates and incubated at 30 °C for 120 h respectively. Cultural conditions such as temperature, pH and storage stability were optimized. Medium I (WSPO; 100:2:3:1, w/w) yielded amyloglucosidase activity 83.92 $Ug^{-1}$and Medium V (WSP; 100:2:3, w/w) resulted into activity 67.64 Ug^{-1} at 48 h optimal time. There was decline in activity after 48 h in all samples but with maximum activity at pH 5. *Aspergillus niger* OTF showed maximum amyloglucosidase activity23.5 Ug^{-1} as compared with *A. flavus* CLOR1 20.07 Ug^{-1} at 30 ºC. The residual activities in term of storage stability were 43.9% and 44.9% at 4 °C and 68. 6% and 27.8% at 28 °C at week 2 respectively which later declined over a period of time. This study revealed that higher glucoamylase activity was significantly enhanced using these inexpensive and readily available agro-residues by both strains of isolates in solid culture medium. Thus, these residues could be a potential and economic source for the production of amyloglucosidase.

Keywords: Amyloglucosidase, Agro-substrates; A*spergillus niger* OTF; *Aspergillus flavus* CLOR1; Solid Culture Medium

INTRODUCTION

Amyloglucosidase or glucoamylase (GA) is an induction enzyme used in food processing industries as well as in commercial purposes for production of glucose from the non-reducing ends of starch. Apart from amylose and amylopectin fractions of starch, other molecules such as maltose, dextrins and glycogen are hydrolyzed by the enzyme, which also acts on the α-1,3 bond **(Kumar *et al.*, 2013).** A production has been increasingly applied by the process of solid state fermentation (SSF).This cultivation technique is simple with low energy requirements, superior productivity, cost effective fermentation media, without rigorous fermentation control parameters, produces less wastewater, requires simpler equipment, avoids foaming and lowers the risks of contamination **(Finore *et al.*, 2014).** Starchy food crops have been reportedly used as substrate for the production of amyloglucosidase, which in turn might leads to shortage of food for human consumption **(Anto, 2006).** However, several authors have exploited the abundance and availability of agricultural residues by microbial means for the synthesis of amyloglucosidase **(Vasudeo, 2010; Lakshmi and Jyothi, 2014; Aliyah *et al.*, 2017).** The conversion of these into economic value biocatalyst might greatly reduce environmental and health hazards.

The amyloglucosidasehas been reportedly produced from wheat bran, paddy husk, rice bran, wheat flour, corn flour, coconut seed flour, coconut oil cake, tea waste and other starch containing wastes (**Wannapeera and Suneerat 2008; Khan and Sachin, 2011; Kumar *et al.*, 2013; Aliyah *et al.*, 2016).** *Aspergillus niger* has been investigated to possess the potential to utilize agricultural wastes such as rice bran for glucoamylase production using solid state fermentation (SSF) as SSF gains recognition over submerged fermentation (SmF) in term of high rate of productivity, less effluent generation and relatively higher concentration of products (**Sarojini *et al.*, 2012**).

The principal application of amyloglucosidase is in the production of glucose, a feedstock for the production of high fructose syrups and ethanol for fermentation industries, baking, and brewing and starch processing (**James and Lee, 1996; Anto, 2006; Kumar *et al.*, 2013)**. The present work focused on the production, and characterization of amyloglucosidase at different temperatures, pH and substrate composition as well as the thermostability and storage methods through solid state fermentation by *Aspergillus niger* OTF and *A. flavus* CLOR1 using readily available and abundance agro residues as cheap source.

MATERIALS

Substrates

Rice bran, Soya bean flour, Corn bran, Palm kernel cake, Oyster shell were purchased from Livestock Feeds Mill, Abeokuta North Local Government Area, Ogun State, Nigeria.

METHODS

Sample Collection

Soil sample was obtained from an Orchard Garden in McPherson University Campus. They were collected in sterile polythene plastic bags and labeled appropriately. These samples were properly mixed together to make a composite samples which were used for the study.

Isolation of microorganisms

Serial dilution was carried out by dissolving 1 g of soil sample in 10 ml sterile distilled water. One ml each of these diluted samples was cultured by pour-plating on Saboraud Dextrose Agar and prepared according to manufacturer's instruction. The plates were incubated at 37 °C for 48 h. Series of sub-culturing was done until pure cultures were obtained and preserved on slant.

Identification of the amyloglucosidase producer strains

Identification of the isolates was performed using morphological means and16S rDNA gene sequence analysis. The pure fungi strain were streaked on SDA agar and incubated overnight at 28 °C. A colony was observed under light microscope.
Genomic DNA extraction was carried out according to the protocol of Zymo Research Fungal DNA Mini Prep™ Instruction Manual and kit. A universal primers; forward primer F-ITS4: 5'TCCTCCGCTTATTGACATGS 3') and reverse primer R-ITS1: 5' TCCGTAGGTGAACCTGCGG 3') was used to amplify 16S rDNA gene **(White *et al.*, 1990)**.The PCR reaction for the amplification of the DNA extracts was performed for 36 cycles while the conditions used were initial denaturation at 96 ºC for 5 mins; denaturation at 94 ºC for 30 s; annealing at 56 ºC for 30 s; extension at 72 ºC for 45 s; final extension step at 72 ºC for 7 mins; and final hold at 10 ºC indefinitely. Nucleotide sequences were determined by the analysis of fluorescently labeled ITS Region products generated by PCR cocktail mix on an AB 373a Strech (short gun) DNA Sequencer. (Applied Biosystems Genetic Analysis Systems, Thermo Fisher Scientific, Waltham, MA USA).Primer (ITS4: 5'TCCTCCGCTTATTGACATGS 3' and ITS1: 5' TCCGTAGGTGAACCTGCGG 3') were used in all sequencing reactions. The obtained sequences were aligned by submitting them to the non-reductant nucleotide database at Genbank using the BLAST search program in order to determine the identity of the isolate (http://www.ncbi.nlm.nch.gov). The Phylogenetic trees were constructed by using Neighbour-joining method of the MEGA 7 package (**Kumar *et al.*, 2016)**.

Effect of Substrate composition on Amyloglucosidase Production

Agricultural substrates (15 g) different composition samples were weighed into separate petri dishes (Table 1), moistened with 25 ml distilled water. The whole content was homogenized using vortex mixer and sterilized at 121 °C for 30 min. The fermentation process was initiated by inoculating evenly the medium with 2 ml spore suspension of *A. niger* OTF and *A. flavus* CLOR1 appropriately. The media were then incubated at 37 °C for 120 h in a static condition.

Enzyme Extraction

Fermented substrate composition (0.5 g) was added to 15 ml acetate buffer solution (pH 5) agitated and mixed thoroughly. The suspension was filtered out and then centrifuged at 4000 rpm **(Gupta *et al.*, 2008).** This solid-free supernatant was used as crude enzyme source for assaying amyloglucosidase activity.

Enzyme Assay

Amyloglucosidase activity was determined by adding 5 ml 0.1% soluble starch; 1ml 0.1 M acetate buffer at pH 5, and 1 ml crude enzyme solution together, thoroughly mixed and incubated at 40 ºC in water bath for 10 min. The reaction was stopped by the addition 0.5 ml dinitrosalicylic acid reagent and heated for 5 min. Absorbance was recorded at 540 nm **(Gupta *et al.*, 2008).** The enzyme was assayed and amyloglucosidase activity was recorded and calculated as follows:

$$\text{Amyloglucosidase Activity} = \frac{\Delta E * Vf * Vs}{\Delta t * € * gds * d} \quad \text{.....Equation 1}$$

Where
ΔE = Absorbance at 540 nm
Vf = Final volume including DNS
Vs = Volume (ml) of amyloglucosidase used
Δt = Time of hydrolysis
€ = Extinction coefficient
gds = Dry weight of the substrate (g)
d = Diameter of curvette
One unit of amyloglucosidase activity is defined as the amount of enzyme that releases 1 umol of reducing sugar, per minute with glucose as its standard.

Characterizations of Amyloglucosidase

The crude amyloglucosidase was subjected to characterization by studying the following effect of pH, temperature and storage stability

Effect of pH

The effect of pH was observed by using different buffers of varying pH, citrate buffer of 3.5, acetate buffer of 4.5 and 5.5, and also phosphate buffer of 6.0, 6.5 and 7.0. The buffers were used to assay the enzyme following the enzyme extraction and assay procedures as described in Section 2.2.5 and 2.2.6 above respectively. The absorbance was recorded using wavelength of 540nm and the activities of the enzymes were calculated using Eqn. 1.

Effect of Temperature

The composite substrate samples were subjected to various temperatures 30, 35 and 40 ºC and incubated for 48 h and then enzyme was assayed using acetate buffer pH 5 following the enzyme extraction and assay procedures as described in Section 2.2.5 and 2.2.6 above respectively. The activities of the enzymes were calculated using Equation 1.

Effect of Storage Stability on Amyloglucosidase

The composite substrate samples were prepared and incubated for 48 h at temperature 35 ºC. The fermented substrates were oven dried at 40 ºC and portions were kept at 28 ºC and 4 ºC to monitor the storage stability on weekly basis. pH buffers 5 was used to assay the enzyme following the enzyme extraction and assay procedures as described in Section 2.2.5 and 2.2.6 above respectively. The absorbance was recorded using wavelength of 540 nm. The activities of the enzymes were calculated using Eqn. 1.

RESULTS AND DISCUSSION

Identification of Amyloglucosidase producing fungi

The isolates were identified based on their morphology and confirmed by molecular characterization as *Aspergillus niger* OTF and *A. flavus*CLOR1. The phylogenic trees shown in Fig. 1 and 2 of *Aspergillus niger* OTF and *A. flavus* CLOR1 respectively revealed their relationship with closely related species. The two fungal sp. previously obtained from an orchard garden in McPherson University were selected for amyloglucosidase producers on agar plates, they both tested positive for the enzyme and were classified as excellent producer. Other isolates have been extensively studied and this correlated with amyloglucosidase produced by these fungi **(Pandey, 1995; Pavezzi *et al.*, 2008; Kumar *et al.*, 2013)**. Inspite of present successes, the task of finding new highly active amyloglucosidase producer remains topical.

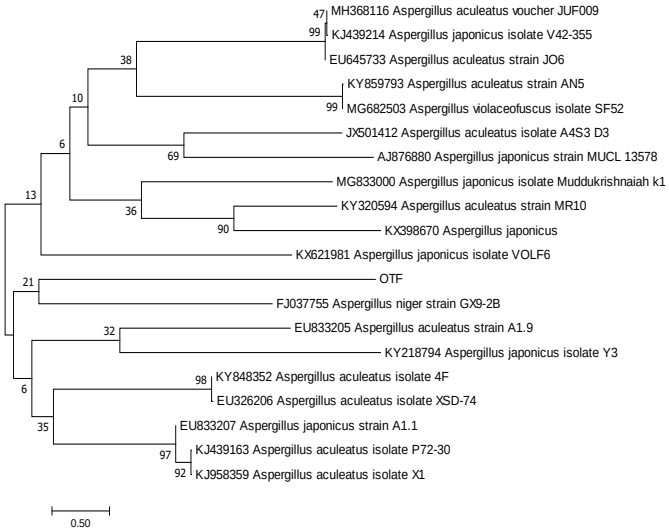


Figure 1 Phylogenetic tree based on gene shows the phylogenic relationship of strain OTF and indicates that it belongs to *Aspergillus niger*. FJ037755 *Aspergillus niger* strain GX9-2B is used as an in group

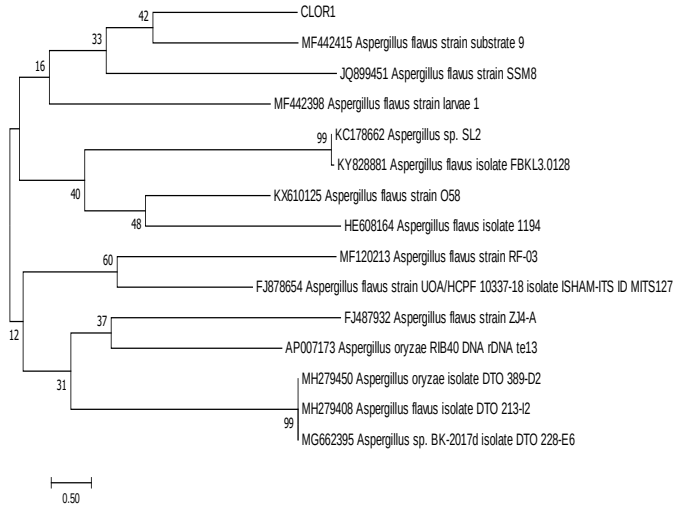


Figure 2 Phylogenetic tree based on gene shows the phylogenic relationship of strain CLOR1 and indicates that it belongs to *Aspergillus flavus*. MF442415 *Aspergillus flavus* strain substrate 9 is used as an in group.

Effect of Substrate composition and fermentation time on Amyloglucosidase production

The effect of substrate composition of agro-residues and fermentation time on amyloglucosidase activities by *A. niger* OTF and *A. flavus* CLOR1 respectively were presented in Fig. 3 (a) and (b). In Fig. 3 (a), Medium I (WSPO; 100:2:3:1, w/w) composting of the largest percentage of wheat bran (carbon source) with oyster shell as source of mineral yielded glucoamylase activity 83.92 $Ug^{-1}$and next was medium V (WSP; 100:2:3, w/w) (67.64 Ug^{-1}) composing same quantity of wheat bran but without oyster shell at optimal fermentation time of 48 h. Medium VI with the highest wheat bran (carbon source) without the mineral source recorded the least activity 27 Ug^{-1} at 48 h. Generally, there was decline in activity of the enzyme after 48 h in all samples.

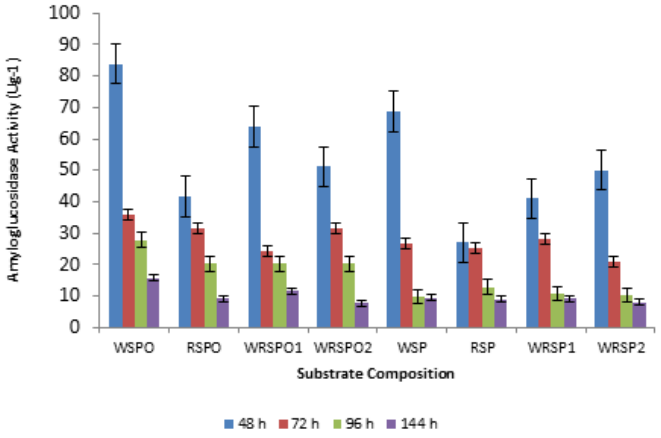
Fig. 3 (b) show that medium V also composting of the largest percentage of wheat bran with oyster shell as source of mineral yielded amyglucosidase activity 65.27 Ug^{-1} and next was medium III at 60.1 Ug^{-1} composing same quantity of wheat bran but without oyster shell at the optimal fermentation time. Medium VI recorded the least activity at 14.9 Ug^{-1}. *Saccharomyces cerevisiae* expressing glucoamylase from *Aspergillus awamori* yielded enzyme activity 0.09 Uml^{-1} **(Flory *et al.*, 1994)** while a recombinant *Saccharomyces cerevisiae* strains Stell7 produced glucoamylase activity 1.5 Uml^{-1} **(Knox *et al.*, 2004)**. *Saccharomyces diastaticus* CL-9in glucose and starch medium resulted into extracellular glucoamylase activity 1.06 Uml^{-1} **(Peres *et al.*, 2006).**This shows that enzymes from these agro-substrates were more economical as compared with starchy substrate reported by these reserachers.

Considering the enzyme yields from the main carbon sources i.e. wheat bran and rice bran, wheat bran produced more enzyme in comparison with rice bran. Wheat bran as the most promising substrate for amyloglucosidase production has been reported by several researchers **(Kaur *et al.*, 2003; Anto *et al.*, 2006; Costa *et al.*, 2007; Kumar *et al.*, 2013)**. Production of very high levels of a hard starch-gel digesting amyloglucosidase under SSF using wheat bran, rice bran, and other components has been reported by **Singh and Soni, (2001)**. However, the enzyme was seen to exhibit its highest activity at 48 h for the both substrates

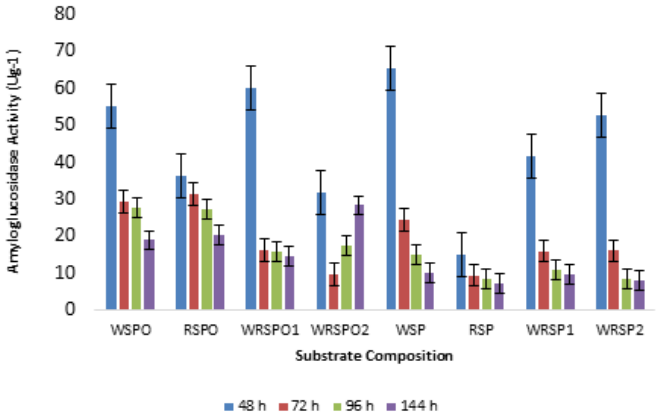
Table 1 Experimented substrate composition of agricultural residues used for amyloglucosidase production by *Aspergillus niger* OTF and *A. flavus* CLOR1

Medium	Sample Code	Wheat Bran	Rice Bran	Soybean Flour	Palm Kernel Cake	Oyster Shell
I	WSPO (100:20:30:1 % w/w)	10	-	2	3	0.1
II	RSPO (100:30:20:1 % w/w)	-	10	3	2	0.1
III	$WRSPO_1$ (50:50:20:30:1 % w/w)	5	5	2	3	0.1
IV	$WRSPO_2$ (50:50:30:20:1 % w/w)	5	5	3	2	0.1
V	WSP (10:2:3 % w/w)	10	-	2	3	-
VI	RSP (10:3:2 % w/w)	-	10	3	2	-
VII	$WRSP_1$ (5:5:2:3 % w/w)	5	5	2	3	-
VIII	$WRSP_2$ (5:5:3:2 % w/w)	5	5	3	2	-

Legend: W- Wheat bran; R- Rice bran; S- Soybean flour; P- Palm kernel cake; O- Oyster shell



(a)



(b)

Figure 3 Effect of substrate composition on amyloglucosidase production by (a) *Aspergillus niger OTF* and (b) *A. flavus* CLOR1 (Error bars with standard error)

Effect of pH on amyloglucosidase production

A. niger OTF and *A. flavus* CLOR1 inoculated into different substrates were incubated at 30 ºC for five days. The enzyme was extracted and the activities of amyloglucosidase produced at different pH were recorded (Fig. 4). The maximum amyloglucosidase activity was obtained at pH 5.0 after 48 h of incubation at 37 ºC. This was very high when compared to other pH range. Optimum pH is very important, the composition of cell wall and plasma membrane of microorganism is known to be affected by the culture pH. Optimization of the culture conditions for amyloglucosidase production by *Aspergillus sp* under SSF and optimum enzyme yield noted at pH 5. It has been reported that fungal amyloglucosidaseacts well in acidic medium **(Norouzian *et al.*, 2006).** Optimum pH for amyloglucosidase from *Aspergillus niger* was 4.8 **(Jafari-Aghdam *et al.*, 2005)** and amyloglucosidase from commercial preparations of *Aspergillus niger* gaveoptimum pH range of 3.5 - 5.0 **(Ono *et al.*, 1998).Vihinen and Mantsala (1989)** also revealed that enzyme produced by *Aspergillus* sp. N-2 exhibited optimum pH in the range of 3.5 - 4.5.

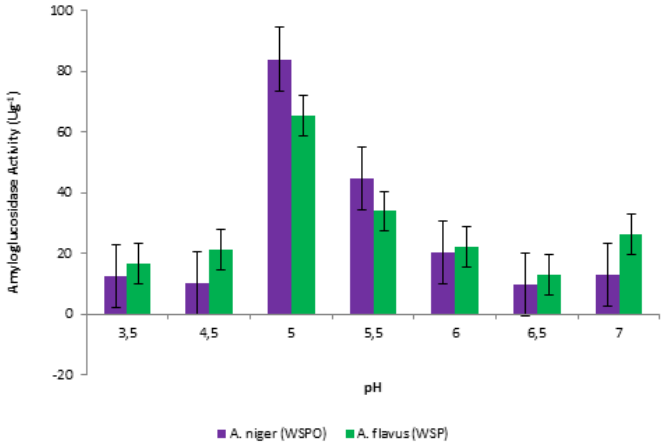


Figure 4 Effect of pH on amyloglucosidase production by *A. niger* OTF and *A. flavus* CLOR1 using WSPO and WSP substrates respectively (Error bars with standard error)

Effect of Temperature on Amyloglucosidase production

Growth temperature is a very critical parameter which varies from organism to organism and slight changes in growth temperature may affect amyloglucosidase production.*Aspergillus niger* OTF and *A. flavus* CLOR1 when incubated at different temperature 30, 35 and 40 ºC, showed optimal yield of amyloglucosidase activity 82.8 Ug^{-1} and 64.76 Ug^{-1}at 30 ºC respectively as shown in Fig. 5. This was contrary to other reports. **Hata *et al.*, (1997)** reported that amyloglucosidase produced from *Aspergillus oryzae* cultivated in solid state fermentation resulted in optimum temperature 56 ºC. Another author described amyloglucosidase from *Aspergillus* sp. HA-2 that recorded optimum temperature 55 ºC **(Anto *et al.*, 2006).**

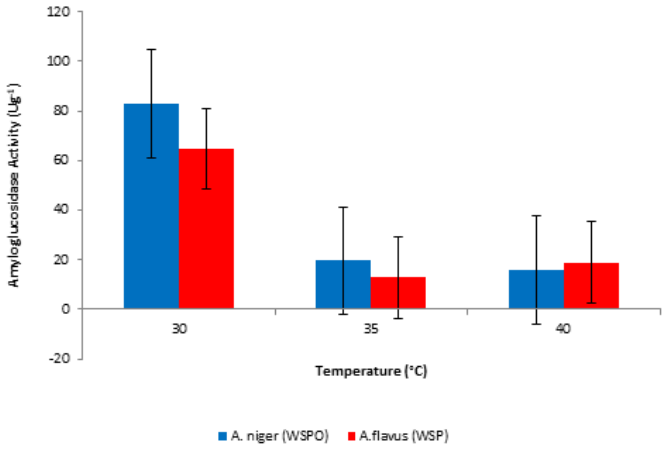


Figure 5 Effect of temperature on amyloglucosidase production by *A. niger* OTF *A. flavus* CLOR1using WSPO and WSP substrates respectively (Error bar with Standard errors)

Effect of Storage Stability on amyloglucosidase production

Cost- and time-effective production of amyloglucosidase was achieved by utilizing dried wheat brans powder as the substrate for production. The effect of storage stability on amyloglucosidase production by *A. niger* OTF and *A. flavus* CLOR1 at 4 °C and 28 °C were presented in Fig. 6. The residual activities of *A. niger* OTF and *A. flavus* CLOR1were 43.9% and 44.9% at 4 °C and 68. 6% and 27.8% at 28 °C in week 2 respectively. However, the activities at both storage conditions declined on weekly basis over a period of five weeks. **Akpan and Adelaja (2004)** reported on stabilization of enzyme preparation that stability is a function of storage time and substrate concentration and also there is a strong interaction between stabilizer concentration and storage time. The results also agreed with observations made by **Tombs (1985),** that enzyme stability is imparted by enzyme substrate. Its high stability ensures efficient utilization under industrial conditions. This work provides a very good platform for the enzyme immobilization studies and scale up production in future.

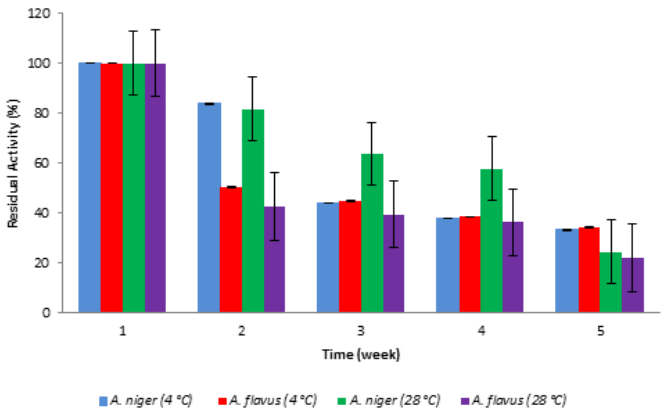


Figure 6 Effect of storage stability on amyloglucosidase production of *A. niger* OTF and *A. flavus* CLOR1 at 4 °C and 28 °C respectively

CONCLUSION

It has been demonstrated that *A. niger* OTF and *A. flavus* CLOR1has the potential to utilize agricultural waste residues for production of amyloglucosidase enzyme. The higher amyloglucosidase activity can be obtained on inexpensive and easily available substrate wheat bran by *A. niger* OTF in SSF. Thus wheat bran residue could be a potential, economic source for the production of amyloglucosidase by solid state fermentation. However, the characteristics of the enzyme provide platform for the enhancement of its industrial applications.

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