British Microbiology Research Journal
5(4): 359-367, 2015, Article no.BMRJ.2015.037
ISSN: 2231-0886

SCIENCEDOMAIN *international*
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Isolation and Screening of Amylase from Moulds Associated with the Spoilage of Some Fermented Cereal Foods

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Authors' contributions

This work was carried out in collaboration between all authors. Author AMO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author GB managed the analyses of the study while authors AOO and TMO managed the literature. searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2015/11190
Editor(s):
(1) Raúl Rodríguez-Herrera, Autonomous University of Coahuila, México.
(2) En Tao Wang, Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México and State Key Laboratory of Agrobiotechnology and College of Biological Sciences, China Agricultural University, Beijing, China.
Reviewers:
(1) Anonymous, Zagazig University, Zagazig, Egypt.
(2) Anonymous, University Nangui Abrogoua, Côte d'Ivoire.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=824&id=8&aid=6862

Original Research Article

Received 1st May 2014
Accepted 18th July 2014
Published 6th November 2014

ABSTRACT

Aims: The aim of the study is to isolate moulds from spoilt samples of three fermented cereal foods (ogi, eko, pap) and screen the mould for amylase production.
Study Design: Primary screening for amylase production by the mould isolates was determined on starch agar. Isolates having the widest zones of hydrolysis were further screened by Solid state fermentation [SSF]
Methodology: Molds were isolated on Sabouraud Dextrose Agar [SDA] incubated at 28℃±2℃ for 72h and identified by standard microbiological methods. Screening of moulds for amylase activity was determined on starch agar medium and flooded with gram's iodine. The isolates were screened

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quantitatively for the production of α-amylase and glucoamylase using Solid state fermentation [SSF] while the enzyme activities were determined spectrophotometrically.
Results: Mean mould population observed on the spoilt food samples ranged from 5.06 log cfu/g for ogi to 5.80 log cfu/g for eko wrapped in leaves. Twenty seven moulds isolated from the samples were identified as *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium citrinum* and *Rhizopus stolonifer*. Majority (70.4%) of the isolated mould produced amylase on starch agar indicated by clear zones around the colonies. Diameter of the zone of hydrolysis ranged from 3.0 mm for *Aspergillus niger* EL4 to 22.0 mm for *Aspergillus flavus* OG1. The highest amylase activity was 30.8% from *A. flavus* OGI while the least was 21.2% from *A. flavus* EN4. Glucoamylase activities observed were between 100 U/ml and 240 U/ml. respectively.
Conclusion: This study contributes to catalogued local fungal isolates that can be used for amylase production and provides additional information to support future research about the industrial enzymes potential of these microorganisms. More work is however needed to determine the optimal production conditions of the enzymes.

Keywords: Glucoamylase; alpha-amylase; mould; spoilt; cereal; pap; ogi.

1. INTRODUCTION

Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents by degrading 1-4 linkage of starch. Amylases are of great importance in fermentation and food industries for hydrolysis of starch and other related oligosaccharides [1,2 3]. Although amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand [4]. Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. Three major classes of amylases such as α-amylase, glucoamylase and β-amylase are reported in microorganisms [5,6].

Alpha amylases [EC-3.2.1.1] degrade α-1,4-glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and alpha limit dextrin [7,8]. Glucoamylase (amyloglucosidase) hydrolyses α - 1, 4 - glycosidic bonds from nonreducing ends of starch molecules, resulting in the production of glucose. Fungal α-amylase and glucoamylase may be used together to convert starch to simple sugars, which in turn serve as a feedstock for production of bioethanol or in the production of high fructose syrups [9].

Traditionally, amylase have been produced microbiologically by submerged fermentation [SmF] and used in one way process in solution, however, solid state fermentation [SSF] holds tremendous potential for the production of enzymes [10]. Solid state fermentation [SSF] has been used in the production of industrial enzymes like amylases, pectinases, tannases, caffeinases, mannanases, ohytases, xylanases and proteases. SSF has great potentials in the developing countries due to its simplicity of operations, low capital cost and high volume productivity [2,1]. Amylases and glucoamylases are produced by various microorganisms including bacteria, fungi and yeast, but a single strain can produce both of these enzymes as well.

Fermented cereals are very widely utilized as food in African countries and a majority of traditional cereal based foods consumed in Africa are processed by natural fermentation and are particularly important as weaning foods for infants and as dietary staples for adults [11].

Ogi is a fermented cereal gruel prepared from maize, millet or Sorghum and marketed as a wet cake wrapped in leaf or transparent polythene nylon [12]. *Ogi* produced from white maize could be boiled to give a thicker consistency, wrapped in leaf or nylon mould, allowed to cool and set to a gel known as *eko* or *agidi*. The wet *ogi* can also be boiled at 8-10% total solids into a porridge or pap which serves as weaning food for infant [13,14].

Generally fermented foods do not get spoilt easily because the by-products of fermentation which also include organic acids, hydrogen peroxide and bacteriocin serve as preservatives against spoilage organisms [15,16]. However, various microorganisms are found to cause the spoilage of *ogi* and *eko*, during storage. Major moulds involved in the spoilage of these fermented foods include *Rhizopus* spp.,

Aspergillus spp. *Penicillium* spp and some yeasts [17,18].

Spoilage of these fermented foods by these microorganisms will involve the production of various hydrolytic enzymes that will enable them utilize the nutrients in the product. Spoilage organisms while growing on food materials have been reported to produce some secondary metabolites including enzymes; amylase is one of such enzymes produced especially on starchy foods. Various moulds are commercially used for enzyme production [19] and it is believed that the ability of these moulds to grow and proliferate, and subsequently cause spoilage will depend on their ability to produce the requisite hydrolytic enzymes to breakdown the fermented foods components. The objective of this study was to isolate spoilage moulds from three traditional fermented cereals foods and screen the isolated mould for the production of alpha amylase and glucoamylase.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

Fresh samples of two cereal based fermented foods: *ogi* and *eko* were purchased from a market in Abeokuta, Ogun State, Nigeria. The *ogi* was sold wrapped in polyethylene nylon while the *eko* was wrapped in polyethylene nylon and leaves of *Thaumatococcus daniellii.*

2.2 Preparation of PAP from Wet *ogi*

Pap was prepared in the laboratory by quick mixing and stirring of the *ogi* slurry with boiling water in a container until a thick gel is obtained. Thereafter, all the samples (*ogi, eko* and pap) were stored at room temperature (28±2℃) until spoilage sets in. During the storage, the samples were observed for change in colour, firmness, texture, odour and appearance of moulds. Portions of samples showing signs of spoilage were taken and used as sources for the isolation of amylase producing moulds.

2.3 Isolation and Enumeration of Amylase Producing-moulds

Each spoilt sample (10g each of *ogi* and *eko;* and 10ml of pap) was homogenized in 90 ml of sterile distilled water. The homogenate was serially diluted and 0.1ml of the selected dilutions was plated out on Sabouraud Dextrose Agar (SDA) using the method described previously [20]. The plates were incubated at 28℃±2℃ for 24-72h. Discrete colonies on the plates were counted and the isolates were purified by repeated sub culturing on SDA. All the mould isolates were stored on SDA slants at 4℃ until required.

2.4 Mould Identification

All the mould cultures were identified and confirmed by studying the morphology of colonies, microscopic examination and characterization using the methods described in Fungi and Food Spoilage [21].

2.5 Primary Screening of Amylase Producing-fungi

The primary screening of amylase production from the isolated moulds was carried out according to the method using starch agar medium and gram's iodine (0.4%KI +0.2% I_2) [22]. Sterilized starch media were inoculated with isolated mould and incubated at 28±2℃ for 24 - 48h; then flooded with iodine solution. A clear zone around fungal colony indicated the production of amylase. For estimating the degradation efficiency, the clear zone surrounding the colony was measured in millimetre using a meter rule.

2.6 Secondary Screening of Mould Isolates

The isolates were screened quantitatively for the production of α-amylase and glucoamylase using Solid state fermentation (SSF). This was carried out in a 1L Erlenmeyer flask containing 50 g rice bran moistened with 50 ml mineral salt solution (tap water; 0.5 g peptone, 0.2 g K_2HPO_4, 0.25 g yeast extract, 0.1g $MgSO4.7H_2O$). The pH was maintained at 6.5. The moist bran medium was autoclaved at 121℃ for 15 minutes and then spread over the inner surfaces of the flasks by vigorous shaking. The flasks were inoculated with 5ml (10^6 CFU) of mould suspension and incubated at 28±2℃ for 120 h.

2.7 Amylase Recovery

The crude amylase produced was recovered using the procedures described previously [23]. To 1 g of the mouldy bran, 10 mL of 0.01 M citrate buffer (pH 4.5) was added and the mixture was shaken on an orbital shaker (LAB-LINE, UK)

at 150 rpm and 28℃ for 1 h. The filtrate was used as crude enzyme source.

2.8 Amylase Enzyme Assay

2.8.1 Measurement of alpha-amylase activity

The α-amylase activities of the isolates were assayed using the routine method [24]. The substrate used was 200 ml of 2% (w/v) starch solution (pH 6.5); 10 ml of the substrate was mixed with 2 ml of each of the crude enzymes solution and incubated at 37℃ for 20mins in a water bath. The reaction was stopped by adding 5 ml of 5 N NaOH solution and stirred for 10 mins. The reaction was neutralized with 2 ml of 4 N HCl and the solution was filled up to 100 ml with distilled water. Aliquots (5 ml) were withdrawn and added to 5 ml of 0.2% I_2- 2% (w/v) KI solution to form the starch-iodine complex. The absorbance for each crude enzyme was measured at 680 nm. The control was prepared using the above method but 2 ml of phosphate buffer was used in place of the crude enzyme solution. Alpha amylase activity was calculated using the formula below:

$$\text{Amylase activity} = 100 \times \frac{(B - A)}{B}$$

Where;

B = Absorbance of blank; A = Absorbance of test

2.8.2 Measurement of glucoamylase activity

The glucoamylase activity of each of the isolates was determined by the reported method [25]. Substrate (1 ml of 2% w/v starch solution, pH 6.5) was mixed with 1 ml of each of the crude enzyme solution and incubated at 60℃ for 60mins in a water bath. The reaction was stopped by adding 2 ml of Di Nitro Salicyclic Acid (DNS). Distilled water (0.7 ml) and 0.3 ml of Rochelle salt was added to each test tube. The test tubes were immersed in boiling water (100ºC) for 5 mins and 5 ml of distilled water were added to each test tube for proper dilution. The absorbance of each enzyme was measured at 540 nm and the resulting glucose liberated was determined from the standard curve for glucose.

One unit of enzyme was defined as the amount of enzyme which liberates 1.0 micromole of D-glucose from starch in 1.0 ml of reaction mixture under the assay conditions.

3. RESULTS AND DISCUSSION

The moulds associated with the spoilage of three fermented cereal products: *ogi, eko* and pap were isolated and screened for the production of amylase enzymes. The changes in colour and mould growth on the samples were observed for 12 days. Mould growth was observed on the pap by the 7th day of storage while wet *ogi* had no noticeable mould growth until the 10th day of storage. *Eko* samples wrapped in leaves had mould growth by day 6 while *Eko* wrapped in nylon had mould growth by day 8. It was reported [18] that *Eko* wrapped in the leaves of *Thaumatococcus daniellii* showed signs of spoilage after 2 days of storage while *ogi* and *pap* had noticeable mould growth within 6-8 days of storage at room temperature.

The mean mould population was 5.06 log cfu/g, 5.51 log cfu/ml, 5.61 log cfu/g and 5.80 log cfu/g for *ogi,* pap, *eko* in nylon and *eko* in leaves, respectively Table 1.

Table 1 presents the samples and the types of moulds isolated from each sample. Twenty-seven moulds were isolated from the spoilt samples and they were identified as *Aspergillus flavus* (5); *Aspergillus niger* (14), *Aspergillus fumigatus* (2), *Penicillium citrinum* (1) and *Rhizopus stolonifer* (5). With the exception of pap*, Aspergillus flavus* and *Aspergillus niger* were isolated from all the other samples tested while *Penicillium citrinum* was isolated only from pap.

The frequency of occurrence of the isolated moulds from the samples is presented in Fig. 1. *Aspergillus niger* had the highest percentage of occurrence (51.9%), *A. flavus* and *Rhizopus stolonifer* both had 18.5% while *Penicillium citrinum* had the least (3.7%).

Carbohydrate rich-foods are often spoilt by fungi especially species of *Rhizopus, Penicillium* and *Aspergillus* [26]. The isolation of these moulds from the fermented food samples is in agreement with the previous report [18] who isolated species of *Rhizopus* and *Penicillium* from stored *ogi, eko* and fufu. The presence of mould such as *Aspergillus niger, Aspergillus flavus, Rhizopus spp.* and *Penicillium* spp. during the spoilage of *ogi* and *eko* also agrees with the previous findings [17,27] who reported that the most important mould that cause spoilage in stored wet *ogi* and *eko* are *Rhizopus nigricans, Aspergillus* and *Penicillium*. However, the incidence of *A. flavus* on the stored fermented

food is of great health concern to the public. *Aspergillus flavus* produces aflatoxin in foods when they grow and are implicated in liver cancer in humans [28,29]. There is therefore need for more concerted efforts at preventing the contamination of these fermented foods by such organisms.

Primary screening of the mould isolates for amylase production on starch agar showed that 19 (70.4%) of the 27 isolates produced clear zones on starch agar. The diameter of the zone of hydrolysis ranged from 3.0mm to 22.0mm Table 2. *A. flavus* OG1 produced the highest zone of hydrolysis (22.0mm). This was followed by 21.00mm by *A. flavus* OG4; 13.00mm by *A. flavus* EN4 and 10.0mm by *A. flavus* OG2. With the exception of *A. flavus* EL3 isolated from *eko* wrapped with leaf, the zones of hydrolysis produced by the *Aspergillus flavus* isolates were significantly ($p<0.05$) higher than the zone of hydrolysis produced by the other mould isolated.

Table 3 shows the mean of the clear zone produced by the mould species. Of the 27 mould isolated from the spoilt samples, 80% of *Aspergillus flavus* and 46.67% of *Aspergillus niger* isolated produced clear zones on starch agar while all (100%) the *Rhizopus stolonifer, Aspergillus fumigatus* and *Penicilium citrinum* isolated produced clear zones on starch agar. The mean clear zone ranged from 16.5±5.9mm for the *Aspergillus flavus* isolates to 4.0±0.0mm for the *Penicilium citrinum* isolate.

Table 1. Mean mould population (log cfu/g or cfu/ml) and the types of mould isolated from each spoilt sample

Sample	Isolate code	Identification of isolate	Mean population (log cfu/g or mL)
Ogi	OG1, OG2, OG4	*Aspergillus flavus*	5.06
	OG3, OG6, OG7	*Aspergillus niger*	
	OG5	*Rhizopus stolonifer*	
	OG8	*Aspergillus fumigatus*	
Pap	PA1	*Rhizopus stolonifer*	5.51
	PA2	*Penicillium citrinum*	
E*ko* in leaves	EL1, EL2, EL4, EL6, EL7	*Aspergillus niger*	5.80
	EL 3	*Aspergillus flavus*	
	EL 5	*Aspergillus fumigatus*	
E*ko* in Nylon	EN1, EN3, EN5, EN6, EN8,	*Aspergillus niger*	5.61
	EN10 EN 2, EN7, EN9	*Rhizopus stolonifer*	
	EN 4	*Aspergillus flavus*	

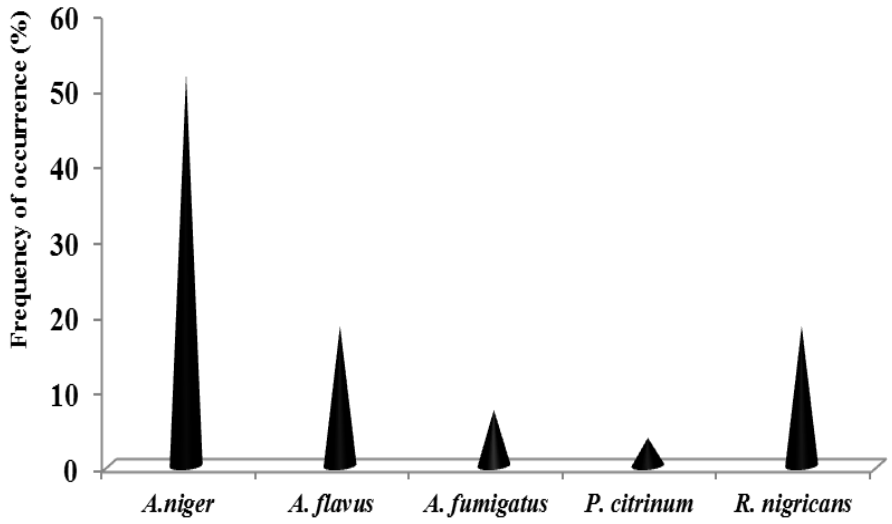

Fig. 1. Frequency of occurrence of moulds in the spoilt *ogi,* pap and *eko* samples

Several studies have confirmed mould species as capable of producing high amount of amylase [1,30,31]. [32] Reported amylase production from *Aspergillus niger***,** *Aspergillus flavus, Aspergillus phoenicis* and *Penicillium granulatum* isolated from garden soil, rhizospheric soil, effluent of textile industry and apple fruit respectively. Similarly [33] isolated *Rhizopus nigricans* which produced significant quantity of amylase in a simple medium of boiled mashed maize.

The amylase activities of the selected mould as presented in Fig. 2 ranged from 17.8% to 30.8%. The highest amylase activity was 30.8% from *A. flavus* OGI, followed by 27.4%, 22.3% and 21.2% from *A. flavus* OG4, *A. flavus* OG2, and *A. flavus* EN4 respectively. Glucoamylase activities of the mould isolates are shown in Fig. 3. The glucoamylase activities observed was between 100U/ml and 240U/ml. All the *Aspergillus flavus* isolates tested, had significantly ($p<0.005$) higher glucoamylase activities as compared to the other isolates.

Table 2. Zone of hydrolysis of isolated mold on starch agar

Isolate	Clear zones (mm)
Aspergillus flavus OG1	22.00
Aspergillus flavus OG2	10.00
Aspergillus niger OG3	7.00
Aspergillus flavus OG4	21.00
Rhizopus stolonifer OG5	8.00
Aspergillus niger OG6	0.00
Aspergillus niger OG7	4.00
Aspergillus fumigatus OG8	7.00
Rhizopus stolonifer PA1	7.00
Penicillium citrinum PA2	4.00
Aspergillus niger EL1	5.00
Aspergillus niger EL2	0.00
Aspergillus flavus EL3	0.00
Aspergillus niger EL4	3.00
Aspergillus fumigatus EL5	5.00
Aspergillus niger EL6	0.00
Aspergillus niger EL7	0.00
Aspergillus niger EN1	7.00
Rhizopus stolonifer EN2	6.00
Aspergillus niger EN3	5.00
Aspergillus flavus EN4	13.00
Aspergillus niger EN5	0.00
Aspergillus niger EN6	4.00
Rhizopus stolonifer EN7	5.00
Aspergillus niger EN8	0.00
Rhizopus stolonifer EN9	6.00
Aspergillus niger EN10	0.00

Table 3. Mean clear zones (mm) produced by different species of mould isolated from the samples tested

Mould isolates	Number tested	No positive (%)	Mean clear zone±SD
Aspergillus flavus	5	4 (80)	16.5±5.9
Rhizopus stolonifer.	5	5 (100)	6.4±1.1
Aspergillus fumigatus	2	2 (100)	6.0±1.4
Aspergillus niger	15	7 (46.67)	5.0±1.5
Penicillium citrinum.	1	1 (100)	4.0±0.0

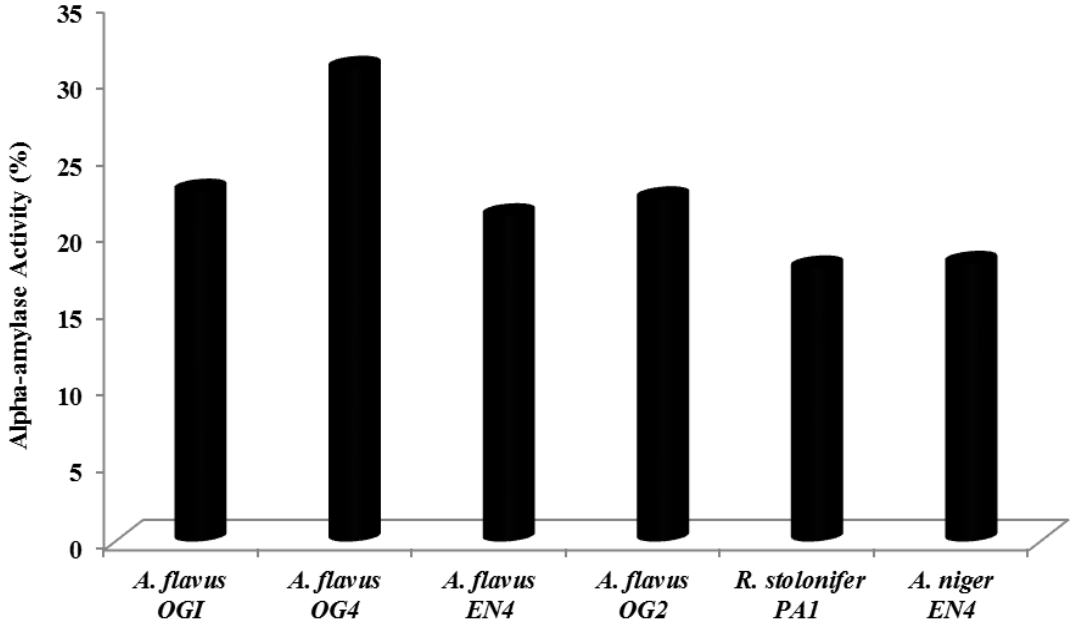

Fig. 2. Alpha-amylase activity (%) of selected mould isolates

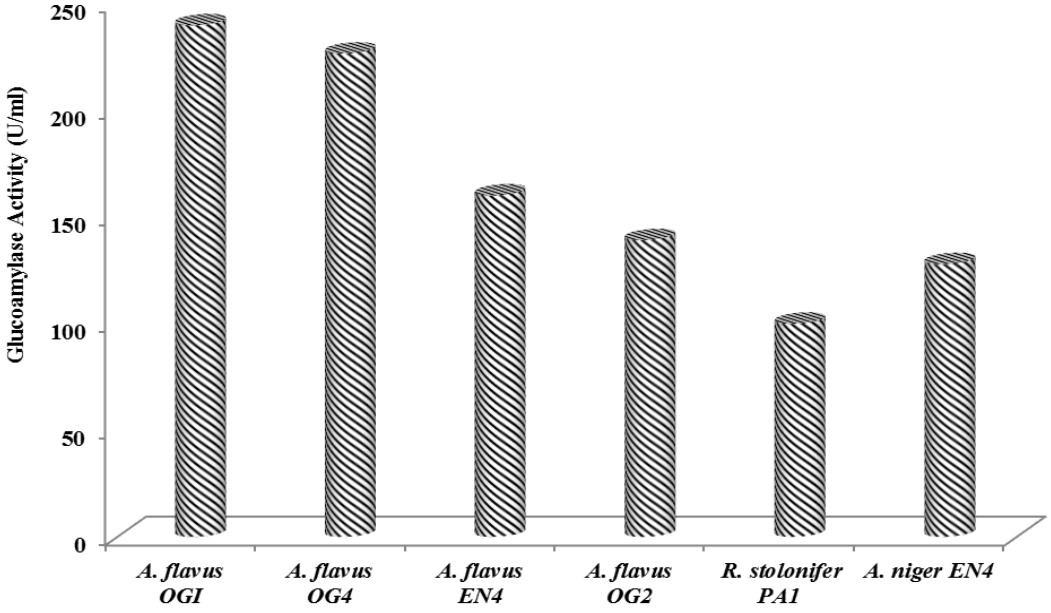

Fig. 3. Glucoamylase activity (U/ml) of selected mould isolates

Most studies on fungal amylase especially in developing country have concentrated mainly on *Aspergillus niger* probably because of their ubiquitous nature and non-fastidious nutritional requirement [34]. However, this study showed that the species of *Aspergillus flavus* isolated from the spoilt fermented food samples produced higher quantity of alpha-amylase and glucoamylase as compared to the species of *Aspergillus niger* and *Rhizopus* tested. This corroborates the study of [35] who reported *Aspergillus flavus* strain that exhibited high growth and amylase production on some polysaccharides including soluble starch

sorghum and cassava peel. Similarly, alpha-amylase enzyme was produced under solid-state fermentation (SSF) conditions from Nile hyacinth by *Aspergillus flavus var. columnaris*.

4. CONCLUSION

In conclusion, this study has shown that moulds associated with the spoilage of *ogi, eko* and pap produce both alpha amylase and glucoamylase enzymes. The *Aspergillus flavus* isolated from *ogi, eko* and pap produced more alpha-amylase and glucoamylase than *Aspergillus niger, Rhizopus stolonifer, Aspergillus fumigatus* and *Penicilium citrinum.* More work is however needed to determine the optimal production conditions of the enzymes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=824&id=8&aid=6862