Research Article

***Cameroon Journal of Biological and Biochemical Sciences* 2020, Vol 28, Serie 2, 131-145**
ISSN 1011-6451/*CJBBS*.2020. Published Online (August 2020) (www.camjournal-s.com)

Production of Collagenases by *Aspergillus terreus* and *Aspergillus flavus* Isolates Using Scales of Tilapia and Croaker Fishes

Production de Collagénase par des Isolats D'Aspergillus terreus et D'Aspergillus flavus à l'Aide d'Ecailles de Tilapia et de Croaker

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Abstract
Collagenase producing microorganisms are crucial for efficient utilization of collagen in food and pharmaceutical industries and fish scales play vital role as inducers. Collagen degrading fungi were isolated from fish scale waste dumps. Proximate analysis of the scales of Tilapia (*Oreochromis niloticus*) and Croaker (*Pseudotolithus senegalensis*) fishes was determined. Ground scales of the fishes were separately used in submerged fermentation for the production of collagenase by isolated fungi. Fermentation conditions were varied and the factors that favored highest yields were combined in a single process. Protein was found to be the highest component of the scales of Tilapia (62.36%) and Croaker (54.29%) fishes. Collagenase activities of 0.326 U/mL and 0.277 U/mL from *Aspergillus flavus* and 0.269 U/mL and 0.245 U/mL from *Aspergillus terreus* were produced respectively on Tilapia and Croaker scales. Activities were optimized at 37 °C, pH 7.5, and 1% substrate concentration in the presence of $CaCl_2$ salts with Tilapia scales resulting in a significant improvement up to 31%. In conclusion, *A. flavus* and *A. terreus* have collagenolytic activity and the scales of Tilapia and Croaker fishes are suitable as substrate for collagenase production.
Keywords: *Aspergillus flavus*, *Aspergillus terreus*, Collagenase, fish scales,

Résumé
Les micro-organismes producteurs de collagénase sont cruciaux pour une utilisation efficace du collagène dans les industries alimentaires et pharmaceutiques et les écailles de poisson jouent un rôle vital en tant qu'inducteurs. Des champignons dégradant le collagène ont été isolés des décharges de déchets d'écailles de poisson. L'analyse immédiate des écailles de Tilapia (*Oreochromis niloticus*) et de Croaker (*Pseudotolithus senegalensis*) a été déterminée. Les écailles du sol des poissons ont été utilisées séparément dans la fermentation submergée pour la production de collagénase par des champignons isolés. Les conditions de Fermentation étaient variées et les facteurs qui favorisaient les rendements les plus élevés ont été combinés en un seul processus. La protéine est la composante la plus élevée des écailles des poissons Tilapia (62,36%) et Croaker (54,29%). Des activités de collagénase de 0,326 U/mL et de 0,277 U/mL *d'Aspergillus flavus* et de 0,269 U/mL et de 0,245 U/mL *d'Aspergillus terreus* ont été produites respectivement sur des écailles de Tilapia et de Croaker. Les activités ont été optimisées à 37 °C, pH 7,5 et concentration de substrat de 1% en présence de sels de CaCl2 avec des écailles de Tilapia, ce qui a entraîné une amélioration significative jusqu'à 31%. En conclusion, *d'Aspergillus flavus et d'Aspergillus terreus* ont une activité collagénolytique et les écailles de Tilapia et de Croaker sont appropriées comme substrat pour la production de collagénase.
mots clés: *Aspergillus flavus*, *Aspergillus terreus*, collagénase, écailles de poisson,

INTRODUCTION

Collagens are proteins that form the major fibrous element of cartilage, skin, ligaments, tendons, blood vessels and teeth and are found in almost all animals. Approximately 30% of human protein comprise of collagen which are mostly found in the connective tissue (Suphatharaprateep *et al*. 2011). Collagen has a rigid structure comprising of triple helix polypeptide fibrils and its degradation is restricted to a few proteases (Di Lullo *et al*. 2002) known as collagenases.

Collagenases are enzymes which degrade the polypeptide backbone of native collagen under conditions that do not denature the protein (Raskovic *et al*. 2014). Collagenases have been isolated from a large variety of organisms including the digestive tracts of fishes, tadpole, land snail, tropical shrimps, certain plants and microorganisms (Wanderley *et al*. 2017). However, due to its biochemical diversity, amenability to genetic manipulations and comprehensive substrate specificities, collagenases of microbial sources are most preferred. Most commercial production uses *Clostridium histolyticum* although many other bacteria such as *Bacillus cereus, B. pumilus, B. licheniformis* and *Klebsiella pneumonia* are known sources of the enzymes (Daboor *et al*. 2010). In addition, filamentous fungi of the genera *Aspergillus, Cladosporium, Penicillium* and *Alternaria* are reported sources of collagenases (Yakovleva *et al*. 2006). High productivity, low production cost, rapid development and extracellular secretion of collagenases by filamentous fungi give them advantage over other microbial sources (Lima *et al*. 2011).

Products of collagen degradation as well as collagen peptides are reported to have some biological activities of industrial and medical interest. They are used as dietary materials, immunotherapeutic agents, cosmetics moisturizers etc. Collagenases particularly of microbial origin play critical roles in morphogenesis, tissue remodeling, embryo development, wound healing and in human diseases such as cancer, arthritis and atherosclerosis (Abdel-Fattah 2013), gastric ulcerations, hypertension and osteoporosis (Hayet *et al*. 2011).

Many researchers have reported production of collagenase in submerged fermentation with the use of supplements in culture media as inducers. Native collagen, gelatin/collagen hydrolysates, malt extract with 1% gelatin and soybean flour are some of the supplements used in culture media for the production. Also, crude substrates such as fish scale powder, fish skin, mammalian, shrimp and crab by-products which are considered as wastes are used as sole carbon and nitrogen sources in media for collagenase production (Guarav and Suresh 2016). Use of wastes has the advantage of keeping the overhead cost of production low in addition to controlling hazards and nuisance it poses on the environment.

Fish scales are composed mainly of collagen which could be modified for various biomedical application including wound healing (Shalaby *et al*. 2020). Fish scales forms a large chunk of wastes generated during fish processing and are majorly unused. Type 1 collagens were identified in scales of Tilapia (Huang et al. 2016) and Croaker fishes (Sampath & Nazeer 2011). These are two commonly available scaly fishes in Nigeria. Fish merchants dispose scales indiscriminately into nearby streams and refuse dumps causing environmental nuisance, water pollution and potential danger to animals inhabiting the streams. Utilization of the scales of these fishes as inducers of collagenases in microorganisms which is the focus of this study should serve both as a form of waste management and in wealth creation.

MATERIALS AND METHODS

Processing of Fish Scales and Proximate Analysis

Freshly removed scales of Tilapia (*Oreochromis niloticus*) and Croaker (*Pseudotolithus senegalensis*) fishes were thoroughly washed and dried in an oven at 50°C for 8 hours. The dried scales were milled to powder using a mechanical grinder and stored in an airtight container at 4°C until use. The moisture content, crude protein, crude lipids, ash, crude fibre and carbohydrate contents of the fish scales were determined following standard methods (AOAC 2002). Briefly, moisture content was determined by drying in oven at 105ºC and weighing intermittently until constant weight. Moisture content (%) = loss in weight of sample/initial weight of sample × 100. Crude protein was determined using the Kjeldahl's method. Crude protein (%) = % Nitrogen obtained × 6.25. Lipid content was determined using Soxhlet apparatus while ash content was obtained by burning the samples in electric furnace. Ash content (%) = weight of ash/weight of sample ×

100. Crude fibre was determined using gravimetric method to measure the remaining organics in the sample after sequential digestion with 0.255N H_2SO_4 and 0.313N NaOH; drying in oven at 104 ºC to constant weight and burning in furnace at 600 ºC for 3 hours. The total carbohydrate in the samples was calculated as Carbohydrate (%) = 100 – (%Crude protein + %Moisture content + %Lipid content + %Ash content + %Crude fibre).

Isolation of collagenolytic fungi

Collagen-degrading fungi were isolated from soil samples collected from fish scale dumpsite. The growth medium used for the isolation comprised of 0.5% (w/v) powdered fish scale in mineral salts solution: $MgSO_4 \cdot 7H_2O$ (0.025 w/v), K_2HPO_4 (1.5 w/v), $FeSO_4 \cdot 7H_2O$ (0.015 w/v), $CaCl_2$ (0.025 w/v) and 1.5% agar powder (TM Media, India) (Wanderley *et al*. 2016). Organisms were isolated using the spread plate technique after suspending the soil sample in sterile distilled water. Plates were incubated at 25 ℃ for 10 days.

Screening for collagenase producing fungi

The collagenase activities of the fungal isolates were confirmed using a plate screening method of Medina & Baresi (2007). The medium comprised of (% w/v): Gelatin (0.5), $MgSO_4 \cdot 7H_2O$ (0.025), K_2HPO_4 (1.5), $FeSO4 \cdot 7H_2O$ (0.015), $CaCl_2$ (0.025) and agar (1.5). After setting, agar plug (6 mm) was removed from the center of each plate and replaced with a plug from a 3 day-old fungal isolate. The plates were incubated at 25 ºC for 72 hours. Plates were flooded with 30% (w/v) Trichloroacetic acid (TCA) for 5 minutes and drained off. Halo zones around the colonies were measured in five planes using meter rule and the statistical mean was taken to indicate extracellular collagenase activity.

Characterization and Identification of Collagenolytic Fungi

All isolates were characterized based on their colonial and cellular morphology while two of them with highest halo zones, used for fish scales hydrolysis were further characterized by molecular techniques. Genomic DNA was isolated using the ZR bashing™ lysis tube and the small sub-unit rRNA genes amplified using Polymerase Chain Reaction (PCR). Primers used were ITS4: 5-TCCTCCGCTTATTGATATGC-3 and ITS5: 5-GGAAGTAAAAGTCGTAACAAGG-3. The PCR products were purified and sequenced using the BigDye Terminator Cycle Sequencing Kit. Translated nucleotide sequences were analyzed for similarities using Basic Logical Alignment Search Tool programme on NCBI.

Production of collagenase

The inoculum was prepared by aseptically washing spores of 5 day old fungi on Potato Dextrose Agar (PDA) slants into sterile distilled water containing 0.1% (v/v) Tween 80 (Wanderley, *et al*., 2016). The spores were enumerated using Neubauer haemocytometer (Weber Scientific Instruments England, Model 3048-12) and adjusted to approximately 3.2×10^5 spores/mL.

Collagenase production was induced in a mineral salt medium comprising of (% w/v) $MgSO_4 \cdot 7H_2O$ (0.025), K_2HPO_4 (1.5), $FeSO_4 \cdot 7H_2O$ (0.015), $CaCl_2$ (0.025) and 1% of micro nutrient mineral solution of $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot H_2O$, and $CaCl_2 \cdot H_2O$ supplemented separately with 0.5% each of the powdered scales of Croaker and Tilapia fishes (Lima *et al*. 2011; Wanderley *et al*. 2016).

Spores of the fungi were separately inoculated into 250 mL Erlenmeyer flasks containing 100 mL of the fermentation medium. Submerged fermentation was carried out at 28 ± 2ºC for 8 days in a shaker incubator at 150 rpm and initial pH of 7.5. Samples (2 mL) were withdrawn at 24 h interval, centrifuged at 10,000 × g, 4°C for 10 min and the supernatants, taken as crude enzymes were analyzed for collagenase activities.

Assay for Collagenase Enzyme

The collagenase assay was carried out on the Azo dye-impregnated collagen (Azocoll, Central Drug House Ltd., India) according to a modified method of Chavira *et al*. (1984). Azocoll (0.5 g) was washed and suspended in 0.05 M Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$. Subsequently, 150 µL each of the crude enzyme and buffer solution were mixed with 270 µL of azocoll suspension in a 5.0 mL reaction tube. The reaction tubes were incubated for 1 hour at 37 ºC in a water bath under agitation. After incubation, samples were chilled in ice for 5 minutes to stop the reaction and centrifuged at 10,000 × *g*, 4 ºC for 20 minutes (Model 5804, Eppendorf). The absorbance of the supernatant was measured at 520 nm in a UV-VIS spectrophotometer (Model 752N; Searchtech). One unit of enzyme activity (U) was defined as the amount of enzyme per mL of crude extract that led to an absorbance increase of 0.1 at 520 nm after 1 hour of incubation as a result of the formation of azo dye-linked soluble peptides.

Optimization of Collagenase Production

Fermentation conditions were optimized using the one factor at a time (OFAT) analysis. Samples were withdrawn for analysis daily throughout the period of production; fermentation mixtures were incubated at temperature ranging from 25 to 40 ºC and pH 7.0 – 8.5; substrate concentration was ranged between 0.5 – 1.0% w/v; and metal ions ($CaCl_2$, $MgSO_4$, $CuSO_4$ and $ZnSO_4$) were used as supplements at 5 mM concentrations each. Collagenase activities were assayed in all the samples.

Data Analysis

The data obtained were analyzed using one-way analysis of variance (ANOVA) while multiple comparisons between means were determined by Duncan's multiple comparisons test. Analysis was performed using SPSS. All data were expressed as means of triplicates ± SD and values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Proximate Composition of Fish Scales

The proximate analysis of the fish scales revealed a high protein content with the scales of Tilapia having higher composition than Croaker (Table 1). Except for the lipid content, values obtained for the other components were significantly different from available figures in literature (Naqvi *et al*. 2014; Shirin *et al*. 2017). This may be because the sample used in this study was previously dried and may have lost some of the components during the process, while the samples in the literature cited were fresh. The high protein component makes the scales good candidate for collagenase induction in microorganisms serving as inexpensive substrate (Wanderley *et al*. 2017). In their studies, Naqvi *et al*. (2014) and Masood *et al*. (2015) also reported high amount of protein in fish scales. Apart from protein, minerals such as magnesium and calcium carbonates or phosphates and a few trace elements which are also essential for collagenase production by microorganisms are found in fish scales (Yusni 2019).

Organisms

Six fungi with collagenase activities were isolated (Table 2). Three of these belong to the genus *Aspergillus* while the others are *Rhizopus, Penicillium* and *Cladosprium.* These fungi were previously reported as candidates for collagenase production (Yakovleva *et al*. 2006; Mahmoud *et al*. 2007; Ferreira *et al*. 2016; Blieva *et al*. 2018). Two of the isolates that had the highest clearance zones (35.70 mm and 34.30 mm) were used in the subsequent hydrolysis step and were identified as *Aspergillus terreus* EV8, and *Aspergillus flavus* JN-YG-3-5 (Table 3).

Figure 1 shows the phylogenetic tree generated from the sequence analysis using the maximum composite likelihood method. Alignment of sequence relative to other reference isolates, for the analysis, yielded two ancestor clades. These clades were distinctively between *Aspergillus terreus* and *Aspergillus flavus* with each clade further leading to polyphyletic branches. While both isolates existed in different clade branch, the evolutionary distance is not too wide or highly significant and therefore reflects a high similarity between both sequences. This will suggest a collagenolytic fitness selection in both ancestor and progeny isolates.

Collagenase activity of fungal isolates

Scales of the Tilapia fish induced more collagenase activity in each of the two fungi and compared favorably with pure collagen used as control (Table 4). The higher protein content of Tilapia scales may be responsible for this since collagen is the major protein of fish scales. The mineral richness of fish scales may also account for its ability to induce more collagenase in the fungi than the pure collagen.

Table 1: Proximate composition of Tilapia and Croaker fish scales

Fish scale	Moisture Content (%)	Crude Protein (%)	Crude Lipid (%)	Ash (%)	Crude Fibre (%)	Carbohydrate (%)
Tilapia	6.82±0.30	62.36±0.11	0.31±0.01	4.18±0.25	11.10±0.14	15.23±2.06
Croaker	6.40±0.30	54.29±0.12	0.21±0.16	4.31±0.25	10.63±0.09	24.16±0.70

Values are means of triplicates, ± SD

Table 2: Collagenase producing fungal isolates

Fungal isolates	Zone of hydrolysis (mm)
Aspergillus tereus (S1)	34.3±0.61[b]
Rhizopus stolonifer (S2)	30.3±1.31[b]
Cladosporium sp. (S3)	28.3±1.47[b]
Aspergillus niger (S4)	17.7±0.52[a]
Penicillium sp. (S5)	21.3±1.21[a]
Aspergillus flavus (S6)	35.7±1.54[b]

Values are means of triplicates, ± SD

Table 3: Identity of collagenolytic fungal isolates used for fish scale hydrolysis

Isolates	**Organism**	**Number of Bases**	**Identity (%)**	**Accession Number**
S1	*Aspergillus terreus* EV8	605	98.98	MK108382.1
S6	*Aspergillus flavus* JN-YG-3-5	591	98.47	MG554231.1

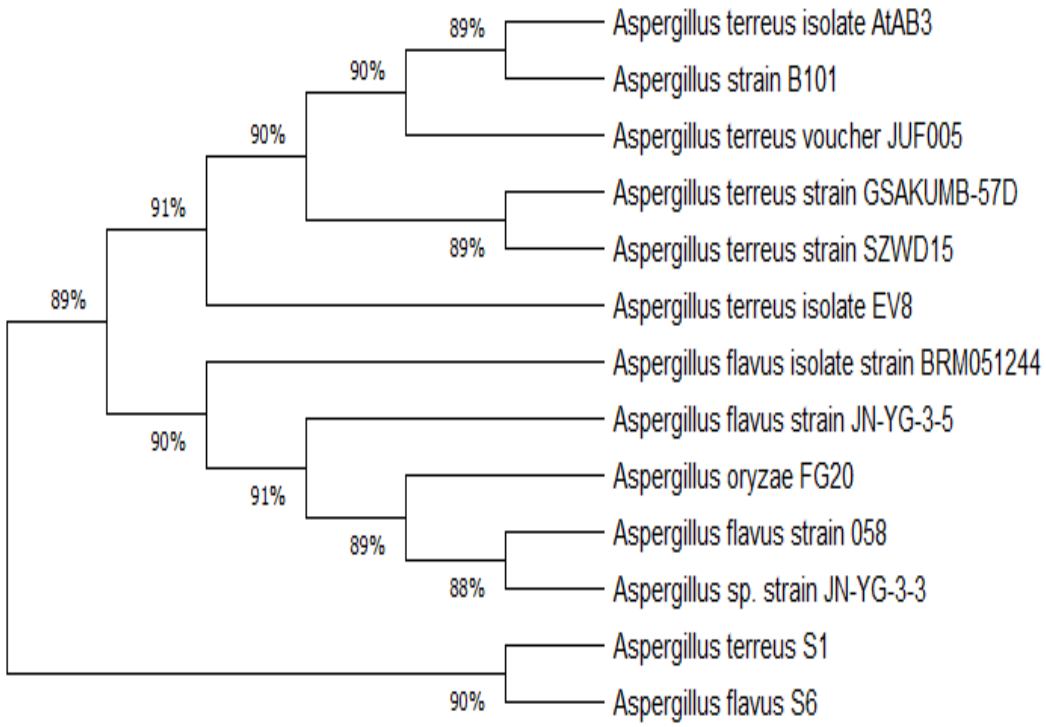


Figure 1: Phylogenetic analysis of isolated fungi using the maximum likelihood method

Table 4: Collagenase activities of *A. terreus* and *A. flavus* after submerged fermentation in media containing Tilapia and Croaker fish scales

Day	Collagenase activity (U/mL)					
	A. terreus			*A. flavus*		
	Collagen	TL	CR	Collagen	TL	CR
1	0.096±0.005^{a}	0.101±0.002^{a}	0.099±0.009^{a}	0.108±0.009^{a}	0.098±0.007^{a}	0.075±0.003^{a}
2	0.158±0.003^{b}	0.167±0.002^{c}	0.149±0.008^{b}	0.150±0.003^{b}	0.178±0.017^{b}	0.136±0.004^{b}
3	0.200±0.003^{c}	0.218±0.007^{de}	0.186±0.015^{c}	0.180±0.002^{c}	0.255±0.017^{c}	0.190±0.003^{c}
4	0.255±0.007^{f}	0.235±0.030^{e}	0.223±0.018^{d}	0.260±0.010^{f}	0.301±0.016^{de}	0.216±0.003^{d}
5	0.237±0.008^{e}	0.277±0.005^{f}	0.245±0.005^{e}	0.249±0.007^{f}	0.326±0.015^{e}	0.269±0.002^{f}
6	0.219±0.007^{d}	0.209±0.005^{d}	0.198±0.008^{c}	0.225±0.007^{e}	0.308±0.016^{de}	0.247±0.003^{f}
7	0.187±0.012^{c}	0.181±0.004^{c}	0.157±0.009^{b}	0.202±0.009^{e}	0.289±0.015^{cd}	0.232±0.003^{e}
8	0.165±0.006^{b}	0.126±0.003^{b}	0.140±0.002^{b}	0.177±0.007^{d}	0.263±0.008^{c}	0.220±0.006^{d}

Means with different superscript in a row are significantly different ($p<0.05$). Values are means of replicates ±SD. *A. terreus* and *A. flavus* were compared separately. TL: Tilapia fish scale, CR: Croaker fish scales

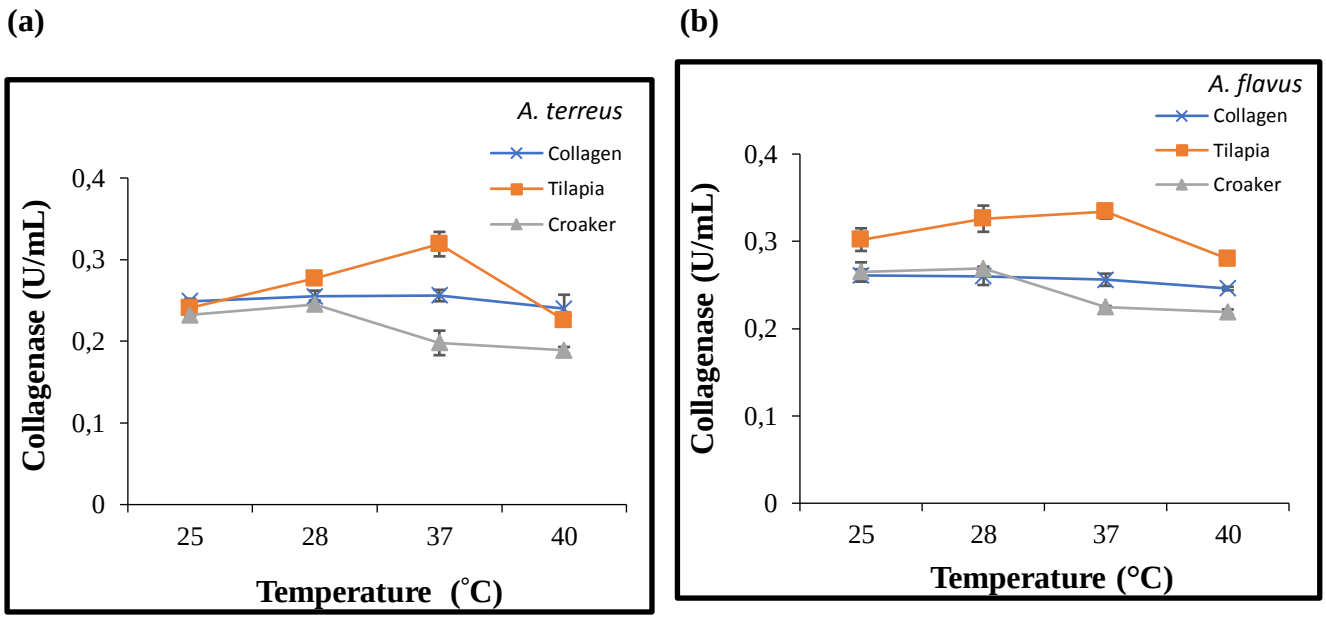
Optimization of Collagenase Production by fungal isolates

Effect of Time of Incubation

Collagenase activity increased with time of fermentation and reached a peaked on day 5 at 0.227 U/ml and 0.245 U/ml from *A. terreus*; and 0.326 U/ml and 0.269 U/ml from *A. flavus* in Tilapia and Croaker scales media respectively (Table 4). Hamdy (2008) and Wanderley *et al*. (2016) also reported optimal collagenase production on day 5 of fermentation while Lima *et al*. (2011) reported 3 days.

Effect of Temperature

Collagenase production was highest at 37°C when the fungi were grown in Tilapia scales medium and 28 °C in Croaker scales. Croaker scales therefore proved to be more efficient in this respect as it compared favorably with the pure collagen. However, although Tilapia induced at a higher temperature, production is much higher than the other two substrates compared. On a cost/benefit ratio therefore, scales of Tilapia may be more favored as inducer of collagenase production than that of Croaker (Figure 2). Anandan *et al*. (2007) stressed the importance of temperature in fungal growth, metabolism, spore formation, germination, enzymatic synthesis and enzyme secretion. At lower temperatures, molecules move slowly and enzymes cannot mediate chemical reactions. As temperature increases, there is rapid movement of molecules, enzyme metabolism is accelerated and cell size is swiftly increased. However, after a certain value, all of these activities occur at overly high rates, and enzymes start to denature (Wanderley *et al*. 2016). Hamdy (2008), Ferreira *et al*. (2016) and Wanderley *et al*. (2016) had previously reported 37 ºC as the optimum for collagenase production.



Values are means of replicates ±SD. Error bars smaller than symbols are not shown.

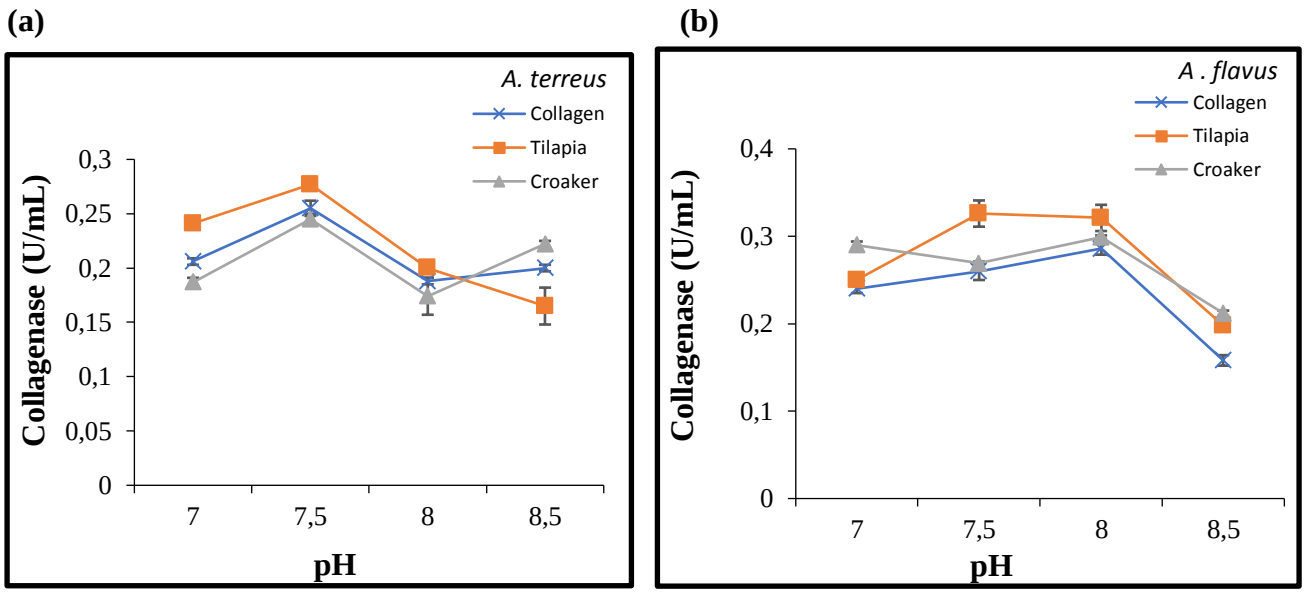
Fig. 2: Effect of temperature on collagenase production by (a) *A. terreus* and (b) *A. flavus*

Effect of pH

Collagenase production by *A. flavus* was highest at pH 7.5 irrespective of substrate used while the optimum for *A. terreus* was substrate dependent and range from pH 7.0, to 8.0 (Figure 3). Optimum pH between 7.0 and 8.0 for collagenase production had been reported previously (Sukhosyrova *et al.* 2003; Ferreira *et al*. 2016) while Gautam & Azmi (2017) reported pH 6.5.

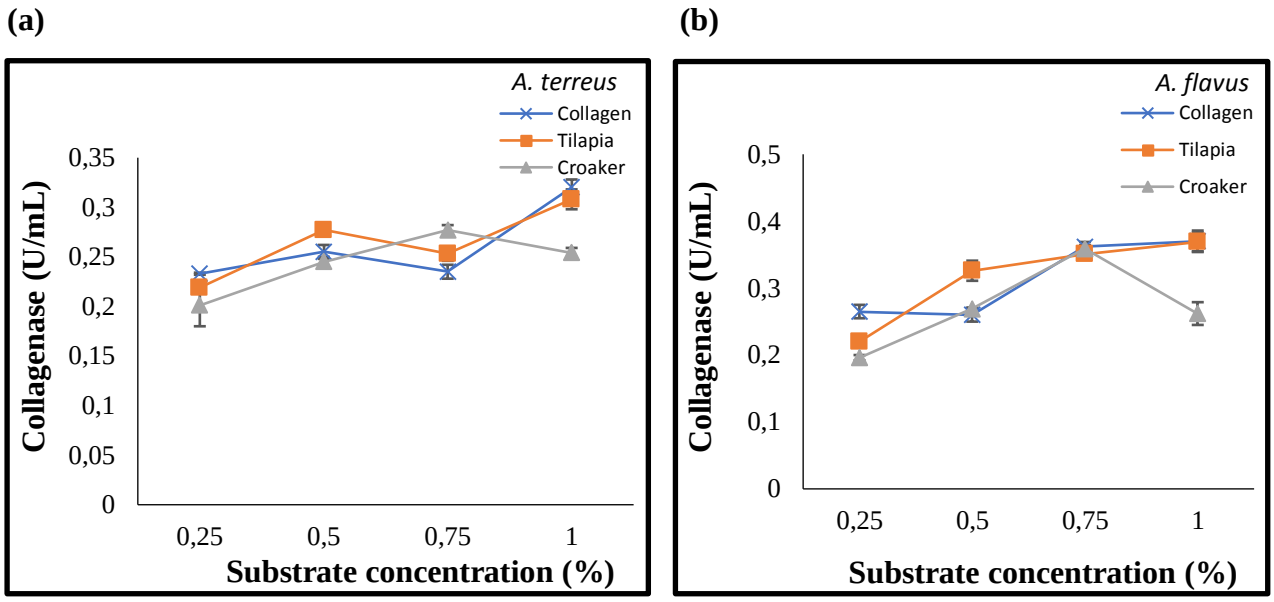
Effect of substrate concentration

The fungi produced collagenase optimally at 7.5% and 1% substrate concentration for Croaker and Tilapia scales respectively (Figure 4). Again, Croaker scales had proven to be more efficient although the amount of collagenase induced by Tilapia scales is higher. Here, the cost/benefit ratio for Tilapia is much higher (3.247 and 2.710) than for Croaker (2.708 and 2.089) scales respectively for *A. terreus* and *A. flavus* which further supports the efficiency of Tilapia scales over Croaker. Suphatharaprateep *et al.* (2011), and Lima *et al.* (2011) also reported optimal collagenase production at 1% substrate concentration. The logic in this is that overcrowding of the active site often lead to non-active site binding resulting in low rate of reaction whereas lower substrate concentrations increases rate of reaction as more substrate molecules collide with enzyme molecules forming more product.



Values are means of replicates ±SD. Error bars smaller than symbols are not shown.

Fig. 3: Effect of pH on collagenase production by (a) *A. terreus* and (b) *A. flavus*



Values are means of replicates ±SD. Error bars smaller than symbols are not shown.

Fig. 4: Effect of substrate concentration on collagenase production by (a) *A. terreus* and (b) *A. flavus*

Effect of medium supplementation

All the mineral salts except $ZnSO_4$ enhanced collagenase productivity (Table 5 and 6). Increase of up to 29% and 25% were obtained with $CaCl_2$ supplementation of Tilapia and Croaker scales media respectively for *A. terreus* while $CuSO_4$ supplementation was most effective at 15% in each of the two substrate for *A. flavus*. These results show that Ca^{2+} and Cu^{2+} had stimulatory effect on collagenase production. The importance of metal ions in aiding enzymes to carry out full catalytic activity had earlier been stressed (Omojasola & Adejoro 2018). Generally, metal ions exist as cations, and are capable of stabilizing or destabilizing the transition state of the enzyme through electrostatic interactions. Metal ions can adhere to a particular number of ligands by accepting free electron pairs to form co-ordinate bonds in a specific orientation (Ferreira *et al.* 2016). Ions of Calcium, Copper, Magnesium and Zinc had been used previously to enhance collagenase production by *Rhizoctonia solani* (Hamdy, 2008), and Ca^{2+} and Mg^{2+} reportedly activated the enzyme in a progressive way up to 10mM. These two metal ions were also reported to enhance and stabilize the collagenase activity of *Bacillus pumilus* Col-J with Ca^{2+} displaying the strongest activation ability. In agreement with our findings, Hanada *et al*. (1973) reported an inhibitory collagenase activity by *Pseudomonas marinoglutinosa* in the presence of Zn^{2+}. In contrast, Park *et al.* (2002) reported that Cu^{2+} inhibited collagenase production from *Scomba japonicas*.

Table 5: Effect of some metal ions on collagenase production by *A. terreus* and *A. flavus* in medium containing Tilapia fish scale

	Collagenase (U/mL)									
	A. terreus					*A. flavus*				
Day	5 mM									
	$CaCl_2$	$CuSO_4$	$MgSO_4$	$ZnSO_4$	Control	$CaCl_2$	$CuSO_4$	$MgSO_4$	$ZnSO_4$	Control
1	0.10 ± 0.01^{a}	0.11 ± 0.01^{a}	0.10 ± 0.0^{a}	0.07 ± 0.01^{a}	0.10 ± 0.01^{a}	0.10 ± 0.02^{a}	0.10 ± 0.0^{a}	0.10 ± 0.01^{a}	0.08 ± 0.00^{a}	0.11 ± 0.01^{a}
2	0.16 ± 0.0^{b}	0.17 ± 0.0^{b}	0.17 ± 0.0^{b}	0.09 ± 0.0^{b}	0.16 ± 0.00^{b}	0.14 ± 0.01^{ab}	0.17 ± 0.0^{b}	0.18 ± 0.02^{b}	0.11 ± 0.00^{b}	0.15 ± 0.00^{b}
3	0.23 ± 0.0^{c}	0.20 ± 0.0^{c}	0.20 ± 0.0^{c}	0.12 ± 0.0^{c}	0.20 ± 0.0^{c}	0.19 ± 0.0^{bc}	0.25 ± 0.0^{c}	0.26 ± 0.0^{c}	0.15 ± 0.0^{cd}	0.18 ± 0.00^{c}
4	0.28 ± 0.0^{e}	0.25 ± 0.0^{d}	0.23 ± 0.0^{d}	0.16 ± 0.01^{e}	0.26 ± 0.01^{f}	0.24 ± 0.00^{de}	0.28 ± 0.0^{e}	0.30 ± 0.02^{e}	0.18 ± 0.0^{ef}	0.26 ± 0.01^{f}
5	0.31 ± 0.01^{f}	0.29 ± 0.0^{d}	0.25 ± 0.0^{f}	0.19 ± 0.01^{f}	0.24 ± 0.01^{e}	0.25 ± 0.02^{f}	0.30 ± 0.01^{f}	0.27 ± 0.02^{e}	0.22 ± 0.00^{f}	0.25 ± 0.01^{f}
6	0.29 ± 0.02^{f}	0.27 ± 0.0^{d}	0.21 ± 0.0^{f}	0.18 ± 0.01^{f}	0.22 ± 0.01^{d}	0.23 ± 0.00^{e}	0.30 ± 0.0^{e}	0.27 ± 0.02^{e}	0.22 ± 0.02^{f}	0.23 ± 0.01^{e}
7	0.24 ± 0.00^{e}	0.26 ± 0.00^{c}	0.16 ± 0.0^{e}	0.15 ± 0.01^{e}	0.19 ± 0.01^{c}	0.20 ± 0.00^{d}	0.30 ± 0.0^{d}	0.24 ± 0.01^{d}	0.20 ± 0.0^{de}	0.20 ± 0.01^{e}
8	0.22 ± 0.0^{d}	0.23 ± 0.00^{c}	0.14 ± 0.0^{d}	0.14 ± 0.01^{d}	0.17 ± 0.01^{b}	0.18 ± 0.01^{c}	0.27 ± 0.0^{c}	0.23 ± 0.00^{c}	0.19 ± 0.00^{c}	0.18 ± 0.01^{d}

Values are means of triplicate determinations (±SD). Means with different superscript in a row for each organism are significantly different ($p<0.05$).

Table 6: Effect of some metal ions on collagenase production by *A. terreus* and *A. flavus* in medium containing Croaker fish scale

	Collagenase activity (U/mL)									
	A. terreus					*A. flavus*				
Day	5 mM									
	$CaCl_2$	$CuSO_4$	$MgSO_4$	$ZnSO_4$	Control	$CaCl_2$	$CuSO_4$	$MgSO_4$	$ZnSO_4$	Control

1	0.10±0.01^{a}	0.10±0.01^{a}	0.10±0.0^{a}	0.09±0.00^{a}	0.10±0.0^{a}	0.11±0.01^{a}	0.10±0.00^{a}	0.11±0.01^{a}	0.07±0.00^{a}	0.11±0.01^{a}
2	0.15±0.0^{b}	0.14±0.0^{b}	0.17±0.0^{b}	0.10±0.0^{b}	0.16±0.0^{b}	0.13±0.0^{ab}	0.15±0.00^{b}	0.20±0.01^{b}	0.10±0.00^{b}	0.15±0.00^{b}
3	0.21±0.0^{d}	0.20±0.0^{c}	0.21±0.0^{c}	0.13±0.0^{c}	0.20±0.0^{c}	0.17±0.02^{c}	0.18±0.01^{d}	0.20±0.00^{e}	0.14±0.00^{c}	0.18±0.00^{c}
4	0.29±0.01^{e}	0.25±0.0^{d}	0.25±0.0^{d}	0.18±0.01^{d}	0.26±0.01^{f}	0.21±0.0^{de}	0.22±0.00^{e}	0.22±0.02^{e}	0.17±0.01^{e}	0.26±0.01^{f}
5	0.30±0.01^{f}	0.27±0.02^{e}	0.26±0.0^{f}	0.21±0.00^{e}	0.24±0.0^{e}	0.24±0.02^{f}	0.27±0.01^{f}	0.25±0.01^{f}	0.19±0.00^{f}	0.25±0.01^{f}
6	0.29±0.00^{e}	0.25±0.00^{f}	0.24±0.0^{f}	0.20±0.01^{e}	0.22±0.0^{d}	0.22±0.02^{e}	0.30±0.01^{e}	0.26±0.02^{e}	0.20±0.02^{f}	0.23±0.01^{e}
7	0.24±0.0^{d}	0.22±0.0^{d}	0.19±0.0^{e}	0.18±0.0^{d}	0.19±0.0^{c}	0.20±0.01^{d}	0.28±0.01^{d}	0.22±0.01^{d}	0.20±0.01^{e}	0.20±0.01^{e}
8	0.20±0.00^{c}	0.20±0.01^{c}	0.18±0.0^{c}	0.16±0.0^{c}	0.17±0.0^{b}	0.17±0.0^{cd}	0.26±0.00^{c}	0.19±0.01^{c}	0.18±0.01^{d}	0.18±0.01^{d}

Values are means of triplicate determinations (±SD). Means with different superscript in a row for each organism are significantly different ($p<0.05$).

Optimized Production of Collagenase

Collagenase activity under optimized conditions yielded 0.427 U/mL; 0.323 U/mL by *A. flavus* and 0.338 U/mL; 0.290 U/mL by *A. terreus* on day 5 in Tilapia and Croaker scales medium respectively (Table 7). This shows significant ($p < 0.05$) higher yields (22% and 18% for *A. terreus*; 31% and 20% for *A. flavus* in Tilapia and Croaker scales respectively) compared to that obtained before optimization (Table 3). In general, *A. flavus* produced higher amount of collagenase while *A. terreus* demonstrated higher efficiency in terms of temperature and substrate utilization. Optimization of collagenase has been studied in different fungal species. Using the one factor at a time (OFAT), Hamdy (2008) reported maximum collagenase activity (212.33 U/mL) by *Rhizoctonia solani* on Saboraud-glucose-collagen medium at 30 ºC, pH 5.5 and supplementation with Cu^{2+} and Mg^{2+}. Lima *et al.* (2011) used the central composite design (CCD) and obtained optimized collagenase of 164 U/mL by *Penicillium aurantiogriseum* on soybean flour at 24 ºC, pH 7.0 and 1.75% (w/v) substrate concentration. It is worthy of note that collagenase activity in this study was higher than that produced by *Pseudomonas* sp CS-20 which was 0.276 U/mL from protein waste (Gautam & Azmi, 2017). In addition, the use of fish scales served a dual purpose of ridding the environment of wastes with its attendant environmental and public health implications, while providing substrate for the production of collagenase.

Table 7: Yield of collagenase by *A. terreus* and *A. flavus* in optimized fermentation condition

Day	Collagenase activity (U/mL)			
	A. terreus		*A. flavus*	
	Tilapia	Croaker	Tilapia	Croaker
1	0.100±0.004^{a}	0.090±0.005^{a}	0.099±0.002^{a}	0.089±0.016^{a}
2	0.150±0.017^{b}	0.176±0.004^{b}	0.168±0.007^{b}	0.162±0.017^{b}
3	0.205±0.002^{c}	0.219±0.009^{c}	0.249±0.016^{c}	0.241±0.002^{c}
4	0.290±0.012^{e}	0.267±0.005^{f}	0.323±0.018^{d}	0.300±0.014^{de}
5	0.338±0.004^{f}	0.290±0.004^{f}	0.427±0.015^{f}	0.323±0.016^{f}
6	0.290±0.003^{f}	0.259±0.002^{e}	0.419±0.016^{f}	0.287±0.014^{e}
7	0.242±0.003^{e}	0.237±0.009^{e}	0.390±0.013^{e}	0.233±0.012^{f}
8	0.200±0.004^{d}	0.205±0.002^{d}	0.327±0.012^{e}	0.209±0.016^{d}

Values are means of triplicate determinations (±SD). Means with different superscript in a row for each organism is significantly different ($p<0.05$).

Conclusion

The results obtained in this study show that scales of Tilapia and Croaker fishes induced production of collagenase in *A. flavus* and *A. terreus.* Tilapia scales induced higher amount of collagenase whereas scales of Croaker were found to be more efficient regarding temperature of fermentation and substrate utilization. The fungi are therefore recommended for their individual advantages. Further studies to improve collagenase activities by combining other fermentation parameters through response surface methodology are also imperative.

ACKNOWLEDGEMENTS

The authors acknowledge support funding from the University of Ilorin 2015 Tetfund IBR Grant

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