

Molecular Screening and Production of α -amylase from Fungal sp

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ABSTRACT

α -amylase producing *Aspergillus* sp. was isolated from decaying bread collected from bakery site located within Enugu metropolis, Enugu state. Standard microbiology, biochemical and molecular techniques (18s DNA sequencing) were used for confirmation of the fungal strain as *Aspergillus tamari*. p-NPG infused nutrient broth confirmed *Aspergillus tamarrii* as prolific producer of α -amylase with rapid yellow colouration after forty eight hours of incubation. Solid state fermentation on rice bran matrix (SSF) system was used for the enzyme production. Cork borer of 2mm diameter was used throughout the production and optimization studies. Carbon sources including: Starch, wheatbran and sugarcane baggase were optimized, starch was found suitable for the protein production with highest α -amylase activity (91.71 $\mu\text{mol}/\text{min}$). Among the nitrogen sources optimized, peptone was found optimal for α -amylase production with activity of 90.34 $\mu\text{mol}/\text{min}$. pH 6.0 was found the best for the enzyme production. Effect of incubation period on the enzyme production showed the 4th day of fermentation as the peak day for α -amylase production from *Aspergillus tamari*. The results from this study have shown that *Aspergillus tamari* among other fungal isolates from mould bread collected from bakery sites in Enugu metropolis has the potential for α -amylase production in a commercial scale for both industrial and clinical applications.

Key words: 18s rDNA, α -amylase, fermentation, Optimization, *Aspergillus tamarrii* T5

INTRODUCTION

α -amylase (1, 4- α -D-glucano hydrolase; E.C 3.2.1.1) is a ubiquitous enzyme responsible for random hydrolysis of α 1-4, α 1-6 glycosidic bonds in polysaccharides. Generally, the amylase family (clan GH-H glycoside hydrolase) is the largest family of the glycoside hydrolase, transferases and isomerases comprising nearly thirty different enzymes specificities (Vijayaraghavan *et al.*, 2011). As described by Vander Maarel *et al.* (2002) the family of the amylase is categorized into four sub groups based on their specificity of their actions and they include: endo, exo, debranching and transferase amylases. Verily α -amylase is widely distributed in plants, animals and microbes, where they play important role of carbohydrate metabolism (Swetha *et al.*, 2006). Amylases from plants and animals suffer many demerits such as: enzyme loads, doubling in production and ease of separations. Microbial amylases are among the most important hydrolytic enzymes and have been studied extensively (Singh *et al.*, 2014). Their ease of separations, short doubling time and much know how technicality in culturing and production makes much very indispensable for vast applications (clinical, industrial and biotechnological bias fields). α - amylases have been reported to be produced by a number of fungi including the family of *Basidiomycete*, *Ascomycete* and the *Deutromycetes*

(Balkan and Ertan, 2005, Nwagu and Okolo, 2011 and Mohammed *et al.*, 2007). Amylase family represents one of the three largest groups of industrial enzymes and accounts for approximately 25–33% of the world enzyme market (Van der Maarel *et al.*, 2002). They are very indispensable in food industries necessarily for starch hydrolysis, clinical for replacement therapy (case of metabolisms and in-born errors), chemical industries e.g detergent, pulp and paper making etc (Mohammed *et al.*, 2007). Search for novel strains of organisms with unique ability for production of amylase has been of great paramount for much advanced biotechnological relevance of the enzyme. Most applications of amylase occur at high temperature and lower pH which are the conditions for starch hydrolysis and liquefaction. ((Goyal *et al.*, 2005; Silva *et al.*, 2005). Most α - amylases are seen denatured at these given physiologic conditions and thus decreases their indispensability; however, novel strains of filamentous fungi especially those of the basidiomycetes and zygomycete families are of thermal tolerant and can withstand relatively low pH condition. Production of α - amylases from these organisms is believed to meet the desired condition required for industrial need of the enzyme. The present study identifies some fungal strains using molecular techniques with unique properties for production of α - amylases with promising enzymatic characteristics.

MATERIAL AND METHODS

All chemicals/reagents used in the present study are of analytical grade and are products of Sigma-Aldrich, Bristol and May and Baker. The analytical equipments are in good working conditions and always calibrated at each use.

Sample Collection

Bread from bakery site was collected within the axis of Enugu metropolis in a clean plastic container. Thereafter it was transported to the laboratory where it was placed in an anaerobic jar for microbial infestations and decay.

Isolation and Identification of Fungal Strains from the Decayed Bread

Strains of fungi isolates were isolated from the decayed bread in a prepared nutrient broth using standard microbiology (culturing and microscopy mounting) and biochemical (sugar fermentations) techniques as described by Ezeonu *et al.* (2013).

Screening of *Fungi* isolates. for α -Amylase Production

Identified *fungi isolates* were screened for α -amylase producing ability using potato dextrose broth supplemented with 2mM p-NPG as described by Gheytaichi *et al.* (2010). The inoculated culture broth was incubated at 37°C for 3 days.

Molecular Identification of *Aspergillus* sp.

Genomic DNA (gDNA) from the selected isolate with high potentials of α -amylase production was obtained using the QIA amp DNA Mini Kit. The 18S rDNA gene was amplified by RT-PCR (the conditions for the amplification stated below) using the forward (5'-GGTTTGATCATGGTCAG-3') and reverse (5'-AGTTACCTTGTTACGACT-3') primers. The amplified DNA sequence was compared to the Gen Bank database of National Center for Biotechnology Information (NCBI) using the BLAST program (Kumar *et al.*, 2016).

Table 1 Conditions for Amplification of the Bacteria Genome using RT- PCR

TREATMENT	TEMPRETURE (°C)	TIME (Min)
Pre-denaturation	90	7
Denaturation	94	1
Annealing	52	1
Elongation	71	7
Final elongation	72	7

Assay protocol for the Enzyme Activity

α -amylase activity was evaluated by assaying for glucohydrolase (GH) activity of the enzyme. This was achieved by measuring the release of glucose from starch solution using 3, 5-dinitrosalicylic acid (DNS) reagent assay method described by Miller (1959). The reaction mixture containing 0.5ml of 1% cassava starch in 0.05M sodium acetate buffer of pH 5.5 and 0.5ml of enzyme solution were incubated for 1 hour. 1ml of DNS reagent was added and the mixture was boiled in a boiling water bath for 10minutes. The reaction was stopped by the addition of 1ml of Rochelle salt solution. Assay was carried out at absorbance of 540nm. One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of one micromole of glucose per minute.

Protein Determination

The total protein content of the enzyme was estimated as described by Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard protein. Absorbance was taken at 750 nm.

Enzyme Production

Solid state fermentation technique was used for α -amylase production as described by Silva *et al.* (2006). Production parameters such as: Incubation period, pH, and basic macro nutrients (carbon and nitrogen) were optimized during the production process.

Optimized Incubation Period

Fermenters (250 ml) containing 100 ml of liquid media optimized for α -amylase production contained: 1% (NH₄)SO₄, 0.4% K₂HPO₄, 1% starch, 0.01% sodium acetate, 01% di-ammonium citrate, 0.05% MgSO₄.7H₂O ,0.2% MnSO₄.4H₂O. The whole setups were sterilized at 121°C/ 15psi for 20 minutes using the electronic autoclave. They were incubated at pH 6.0 and at room temperature (37°C) for seven (7) days on solid matrix using sterilized brans from rice. Samples were drawn at every 24 hours till 18 days, each drawn sample was filtrated using the muslin cloth of pore size 2mM and the filtrate (crude extracts) was used to assay for α -amylase activity.

Effect of pH

As described above, each fermenter containing 100 ml of the liquid production media were incubated at pH of 4.0-8.5 in the range of 0.5 units. Initial pH of the liquid broth was adjusted using 2% HCl v/v and NaOH w/v. They were incubated till the optimal day of α -amylase production.

Effect of Nitrogen sources

Four (4) different nitrogen sources used include: Ammonium sulphate, beef extract and peptone for the production of α -amylase in the liquid medium at 1% w/v concentrations.

Fermentation was carried out to the optimum day of α -amylase production at the best physiologic condition.

Effect of Carbon Substrate

Four (4) different rich source of carbon substrates used include: starch, sugarcane baggase and wheat bran were used for the production of α -amylase in the liquid medium at 1% w/v concentrations. Fermentation was carried out to the optimum day of enzyme production at the best physiologic condition.

RESULTS AND DISCUSSION

Pure strains of fungal isolates on the prepared potato dextrose agar medium. Organismal strains were identified using standard microbiology and biochemical identifications.

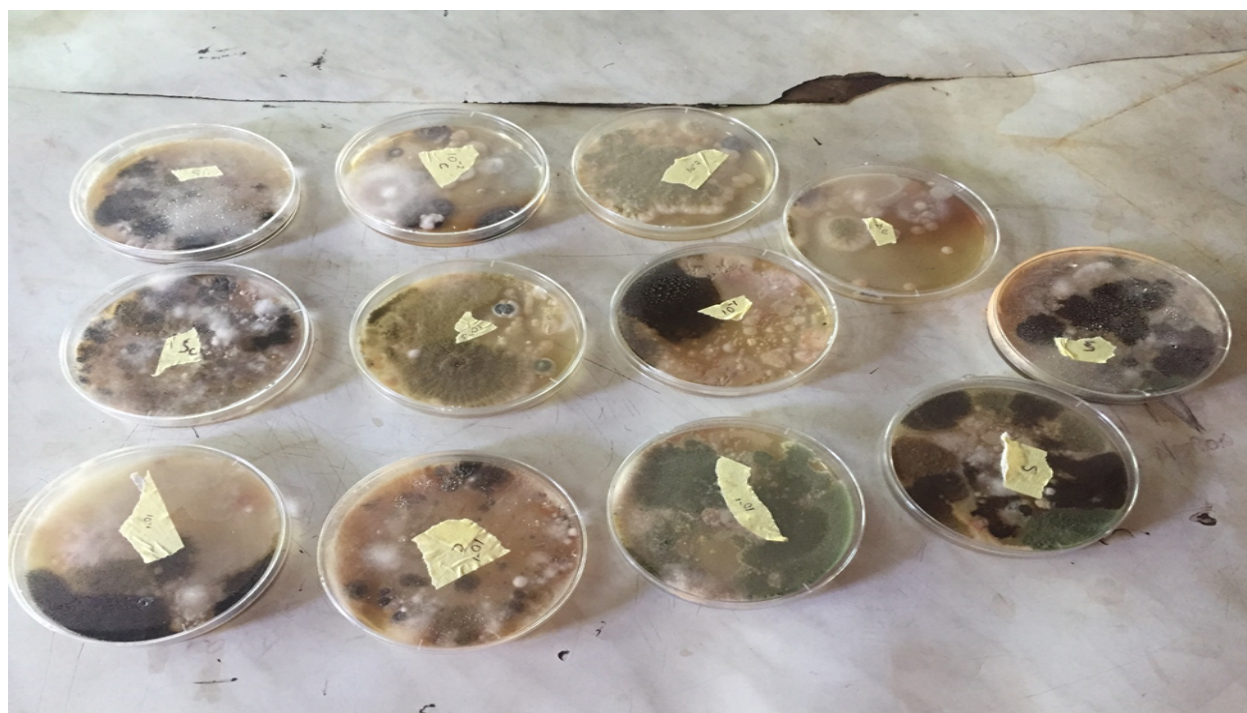


Plate 1. Strains of fungal isolates.

Basic morphological and Biochemical screening were used to identify the isolates as *Aspergillus*, *Monillinia*, *Rhizopus*, *Yeast*, *Penicillium* genre organisms (table 2). Identified fungi isolates from the mould bread were screened using p-nitrophenyl α -Dglucopyranoside to show determine their ability to produce α -amylase. Formation of yellow colouration in the nutrient broth after 48 hours of incubation at 37°C suggests a positive test of only *Aspergillus* sp for α -amylase production (fig 2).

Molecular tests (18s rDNA.) was used to identify the pure isolates of *Aspergillus* Electrophoretogram of the amplified genome of *Aspergillus* using RT-PCR showed a typical base-pair of a fungal strain with 550 bp from the ladder DNA segment (fig. 3). *Aspergillus tamarrii* T5 was identified after the genomic sequencing with ascribed NCBI accession number of KR149638 as shown in the evolutionary relatedness tress (fig. 4).

Table 2 Basic Morphological Features

Sample	Color	Size	Elevation	Shape	Texture	Morphological Characteristics	Suspected fungi
Soil Sample PDA media control	Whitish	Above 7mm	Raised	Irregular	Pluffy Filamentous Mass Mycelium	Well-developed collumela like an umbrella shape.	<i>Mucor</i> sp
Soil Sample PDA Irrigated soil	Creamy/Whitish	1.2mm	Raised	Spherical Regular	Smooth	Non Filamentous Drop-like Separated Colonies	<i>Saccharomyces</i> sp.
Soil Sample PDA irrigated soil 1	Creamy grayish	1.4mm	Raised	Regular	Pluffy	Filamentous spread like	<i>Aspergillus</i> sp.
Irrigated soil 2 PDA	Colorful	1.2mm	Raised	Irregular	Pluffy Filamentous Dark-brown Mycelium	Dark-brown conidia Conidiospore, glubose vesicle	<i>Penicillium</i> sp.
Irrigated soil 3	Blackish, sedentary colouring	1.22mm	Raised curved	Regular	smooth, carved blackish hyphael walls	Conidia linked condiosphores and roundish glubose	<i>Penicillium</i> sp.

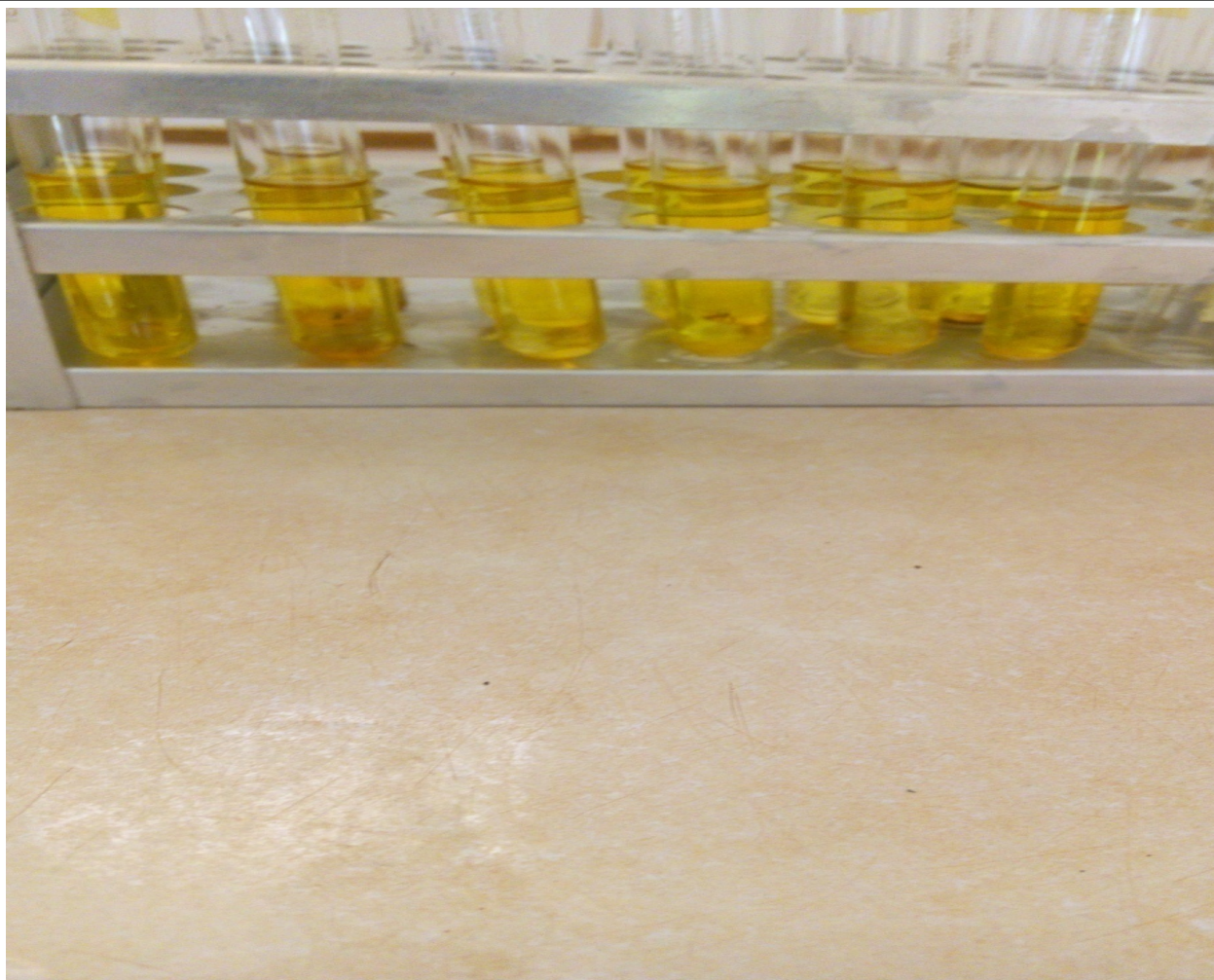


Fig 2 screening of the isolates using p-NPP for α -amylase production.

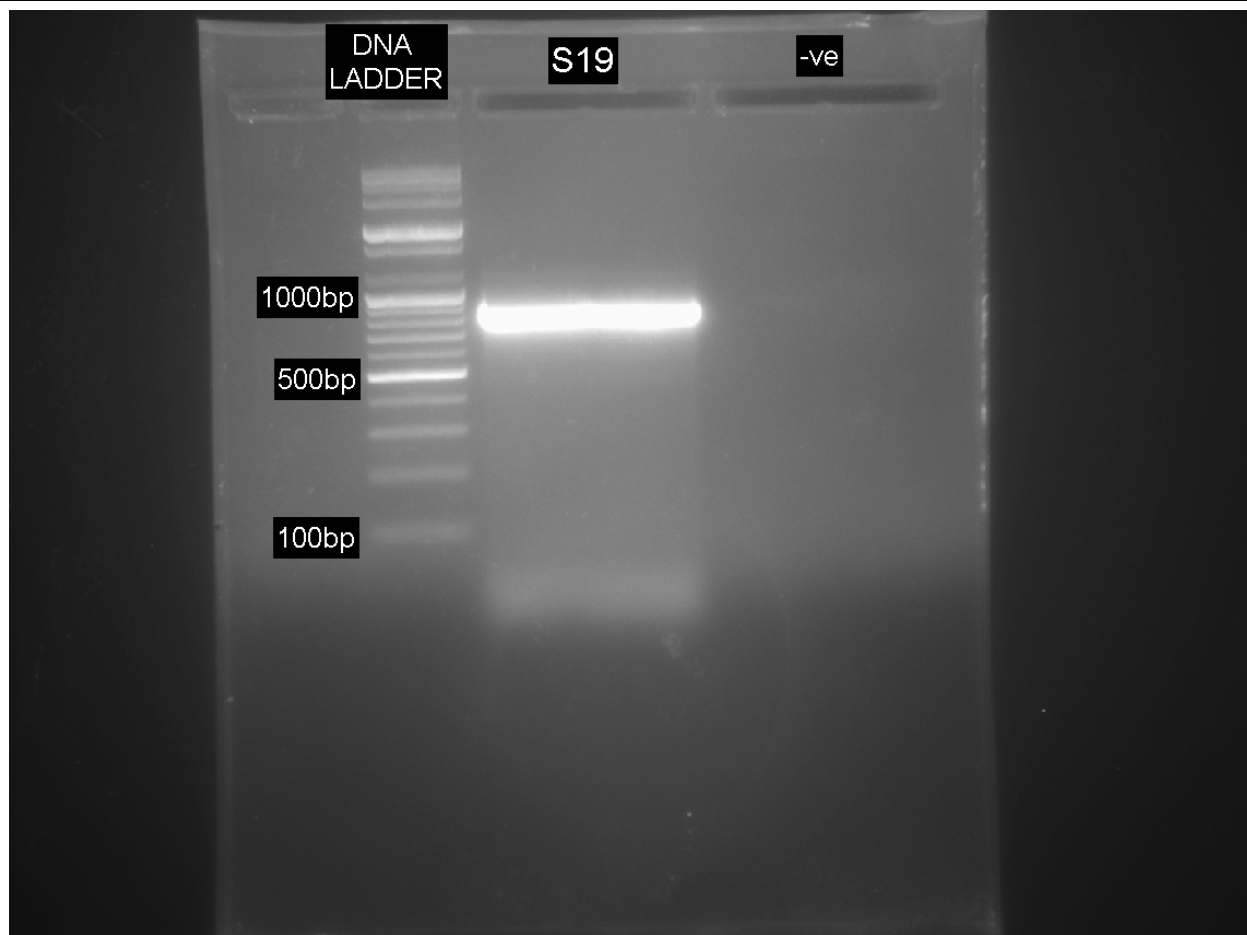


Fig 3 Electrophoretogram of the amplified genomic DNA viewed on a UV trans-illuminator

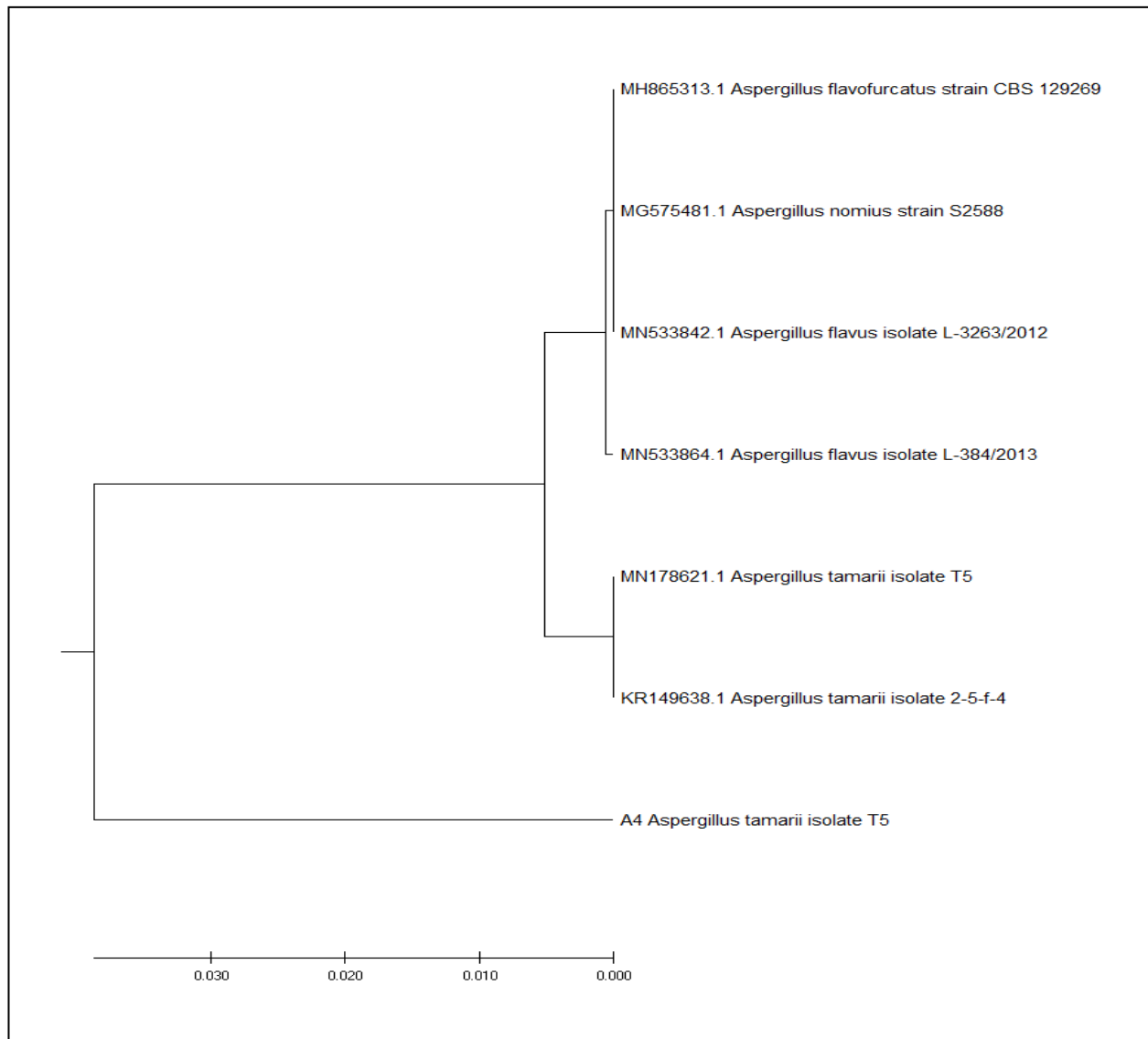


Fig 4 Phylogenetic evolutionary relatedness of strain of *Aspergillus* obtained using NCBI BLAST tools

Studies on effect of incubation period on the production of α -amylase from *Aspergillus tamarrii* sp. showed that the highest α -amylase activity and protein concentration were obtained on the day 4th and 5th of the fermentation periods (Fig 5 and 6). Extracellular protein production at these designated days respectively is evident of catabolite inducement of the substrate present in the fermentation media to the organisms. Closeness in the optimum α -amylase activity and protein concentration is unique to most extracellular protein. The rise and fall in the activity and protein concentrations of the enzyme as reported by Adefila *et al.* (2012) stated that α -amylase are specific to α -1,4 glycosidic linkages in starch and other polysaccharides and its activity diminishes on meeting the branching point (1,6 linkages) in sugars and secondly that this could be attributed to the presence of isoforms of the same enzyme present in the crude extract.

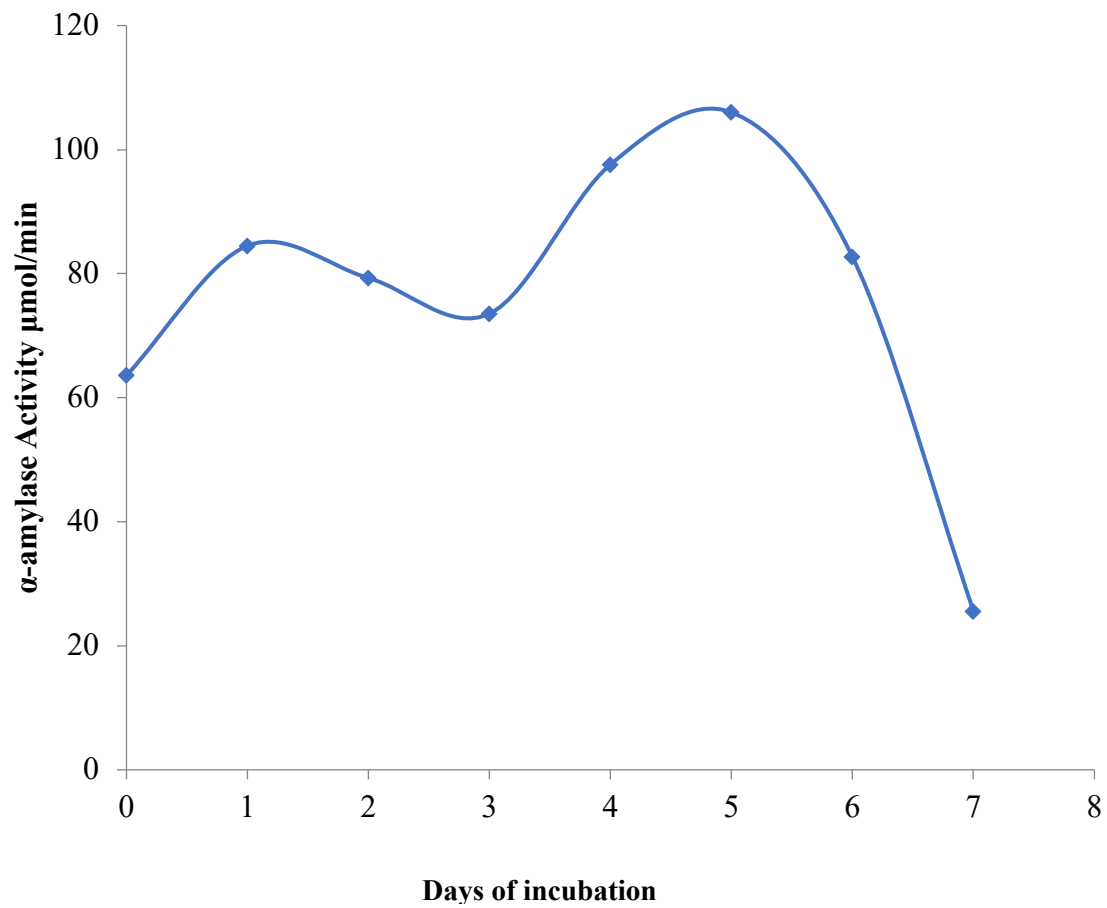


Fig 5 effect of incubation days on α -amylase production

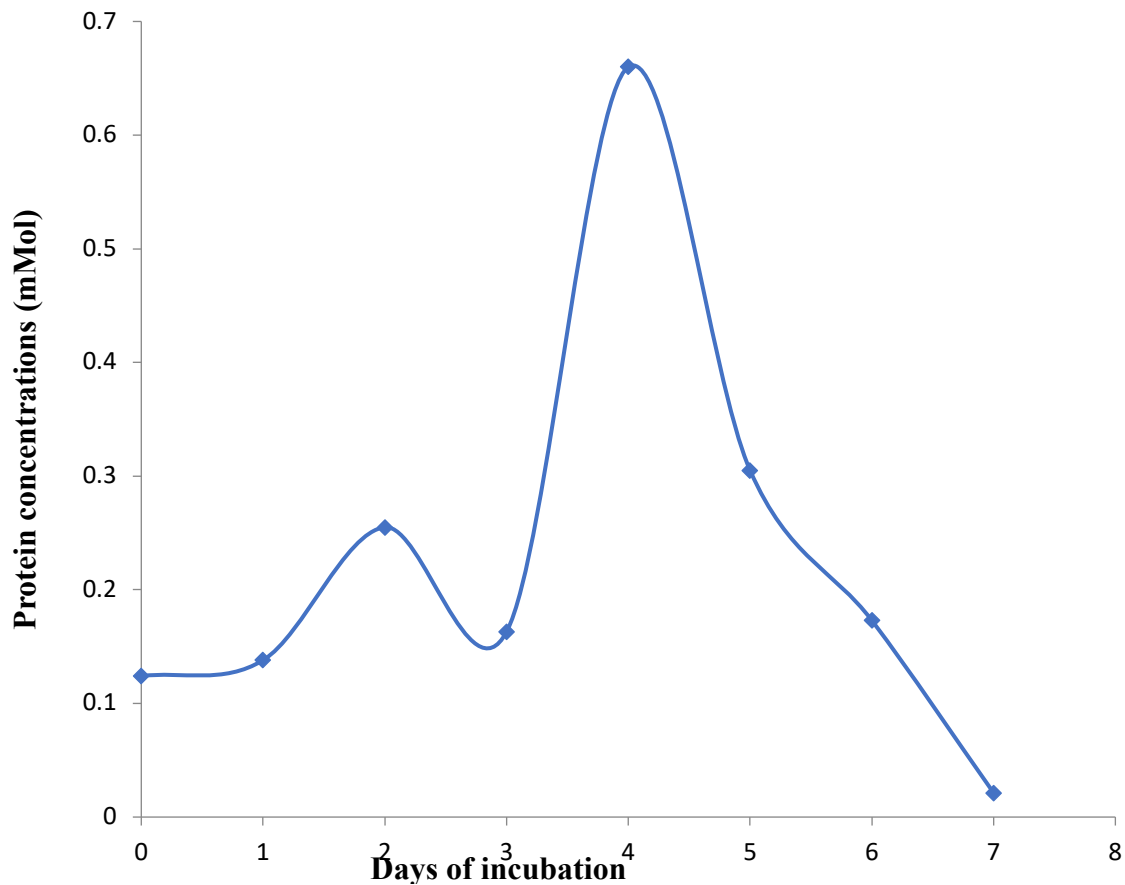


Fig 6. Effect of protein concentrations on the enzyme production

pH is an important physiologic factor to be considered in enzyme production system. It helps to understand the tolerant nature of the organisms and perhaps the extracellular enzyme to hydrogen ion (H^+) concentration of the medium. The optimum pH for α -amylase production was 6.0 (fig. 7). Filamentous fungi are moderate acidophiles and are tolerant to low pH in production system. The report by Shen *et al.* (2012) on α -amylase produced from filamentous *Rhizopus* showed a production optimum at pH of 6.5.

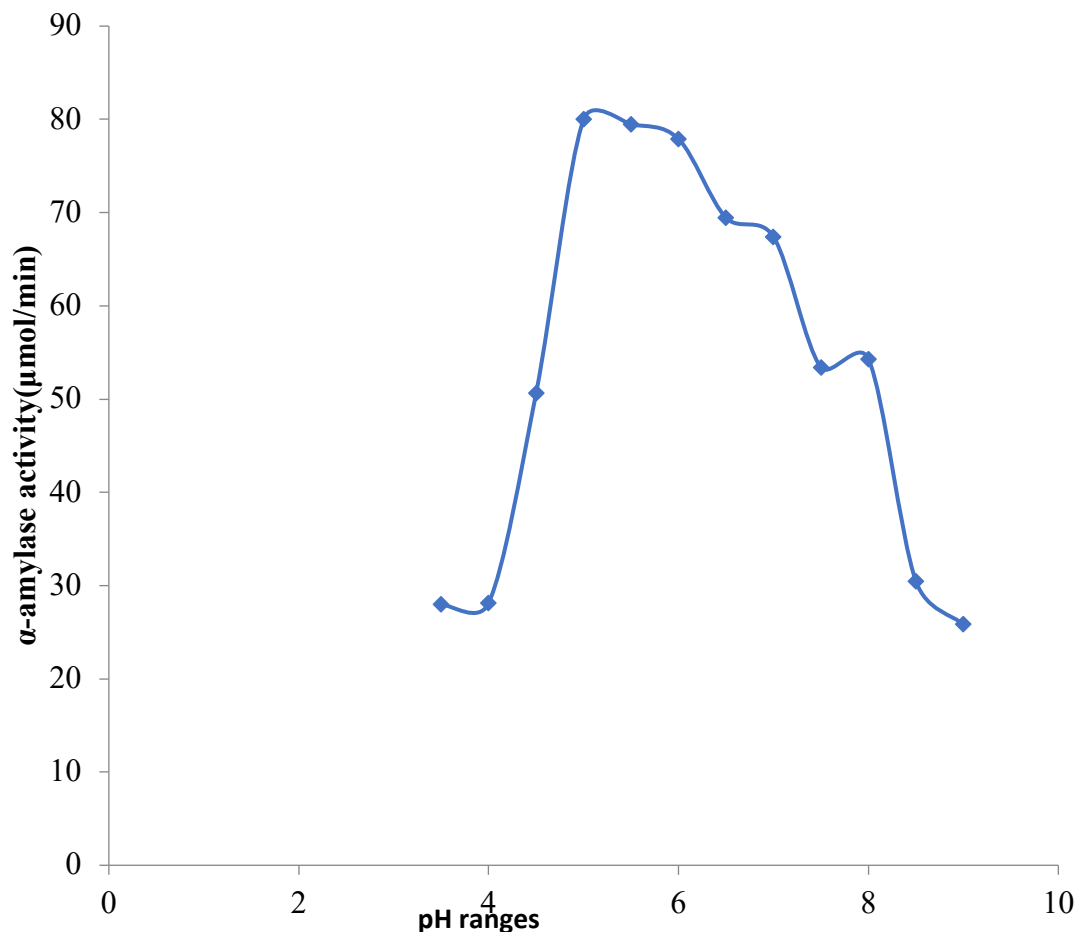


Fig 7 Effect of physiologic pH on α -amylase production from strains of *Aspergillus tamarrii*

Effect of Nitrogen (nutrient) sources on α -amylase production from *Aspergillus tamari* showed that peptone extract served as the best nitrogen for α -amylase production (Fig.8). As reported by Vasiljevic and Jelen, (2001), organic nitrogen sources in fermentation media are super source of amino groups to fermenters in bio-production process. Among the basic nutrient needed for enzyme production, nitrogen, carbon and phosphate radicals serve as important nutrient inducible for bioproduct production from living cells.

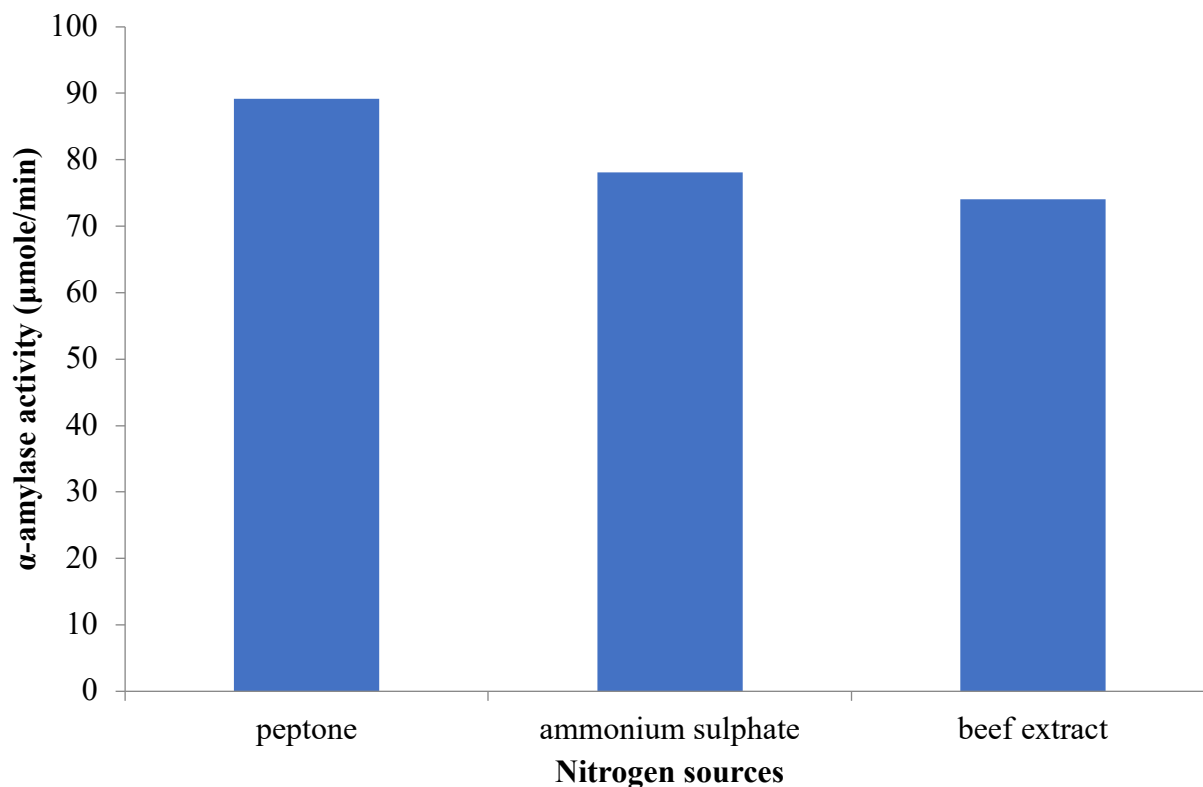


Fig 8. Effect of nitrogen sources on α -amylase production from *Aspergillus tamarrii*.

Studies on the effect of carbon sources on production of α -amylase from *Aspergillus tamari* showed that starch was the best substrate for α -amylase production. Other carbon sources such as sugarcane baggase and wheatbran gave relatively better yield of the enzyme but not upto the effect starch. The preference for starch by the organism for the enzyme production (α -amylase) is evident as starch plays a good catabolite activator for exo α -amylase production as regards to the switching on of *operon* necessary for glucose and oligosaccharide production described by Monod and Jacob (1951). Recall that starch is a plant polysaccharide with repeating glucose moieties of linear α -1-4 and branching 1-6 linkages. The anomeric linkages of the repeating small sugars making up the polysaccharides are specific target of the enzyme. Sudha (2013) reported peak production of α -amylase from *Bacillus* sp. using starch from corn steep liquor as the sole carbon source.

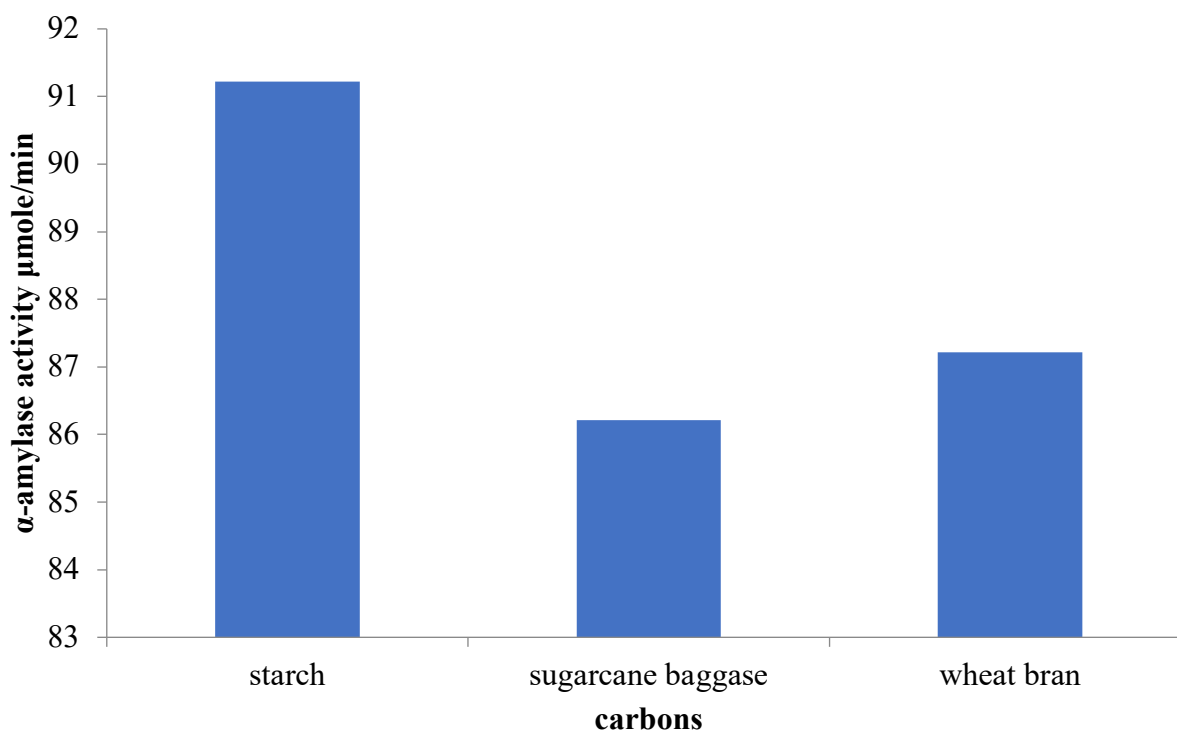


Fig 9. Effect of carbon sources on production of α -amylase from *Aspergillus tamarrii*

CONCLUSION

This research has demonstrated that *Aspergillus tamarrii* isolated from mould bread can be a potential and sustainable source of α -amylase which stands to be an integral part of evolving food and other chemical industries.

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