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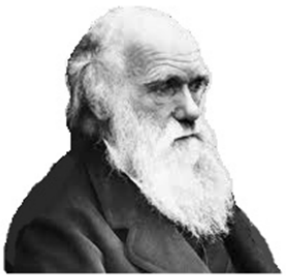

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Isolation and characterisation of alpha-amylase producing yeast from different fermented foods and dairy products

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ABSTRACT

The use of acids in the hydrolysis of starch for industrial and domestic purpose have become relatively unstable, expensive, difficult to control and even harmful to users over the years. This study aimed at isolating and characterizing alpha -amylase producing yeasts and investigate the effects of different cultural and physico-chemical parameters on the production of alpha- amylase. A total of forty-three (43) yeast strains were isolated from different fermented foods such as ogi, wara, palm wine and kunu. The isolates (OG9 and WR4) had the highest activity and were identified as: *Candida tropicalis* strain IFM 63517 and *Candida tropicalis* strain CMC 1836 respectively. Fermentation parameters such as incubation period, pH, temperature, effect of surfactants, carbon and nitrogen source were optimized using submerged fermentation. The optimum pH, temperature and incubation period for enzyme production were: 5.0, 30 °C and 48 hours respectively. Of the carbon sources, soluble starch at 1% concentration was observed to be the best carbon source for enzyme production using the two yeasts. Yeast extract at 1% concentration was ideal nitrogen source for the yeasts. Surfactants Tween-80 was most effective for enhancement of alpha amylase production. $Cacl_2$ supported the highest alpha amylase activity for both strains. This study revealed that favourable fermentation conditions and the selection of suitable growth parameters played key roles in the production of alpha amylase by *Candida tropicalis*. Soluble starch is a good substrate for alpha amylase production. Alpha- amylase producing yeast strains with increasing enzyme activities at high temperature are promising candidates for industrial application.

***Keywords*:** Yeast, *Candida tropicalis,* fermentation, Alpha amylase, soluble starch

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1. INTRODUCTION

Enzymes are biocatalysts used to accelerate biochemical reactions of microorganisms. Apart from their metabolic function, enzymes are used in different industries in the catalysis of many processes such as sugar, pharmaceutical, textile and brewing industries (Maarel van der, 2005).

Currently, enzymes such as protease, amylase, lipases and cellulases have become one of the important detergent formulations in laundry industry. Enzyme formulated detergent is more favourable because it is known to effectively remove stain by degrading the biological component and it is also environmental friendly in terms of reduction in energy and water consumption through lower washing temperature (Burhan et al., 2003).

The development of industrial enzymes has depended on the use of microbial sources, because they can be cultivated economically in short fermentation period and inexpensive media. Hence, the new potential of using microorganism as source of biotechnological and industrial relevant enzymes has stimulated interest in investigation of extracellular enzymes from several microorganisms which include: fungi, bacteria and actinomycetes (Beyer, 2011).

The major classes of microbial enzymes that contribute immediate application are the hydrolytic enzymes, which account for approximately 75% of the industrial enzymes produced (Kirk et al., 2002).

Amylase is one of the most important enzymes which cover around 30% of total commercial enzyme in the world market (Gupta et al., 2003). Earlier, amylase was mainly used in the bakery and brewing processes, nowadays more and more applications of α-amylase are being explored, and one of them is used in the detergent formulation. Currently, almost 90% of all liquid detergents contain amylase (Crab and Shetty, 1999). Amylase breakdown starch to produce smaller products which include: dextrins, oligosaccharides and glucose molecules (kirk et al., 2002).

The three types of amylases: α-amylase, β-amylase and glucoamylase can be produced from fungi, yeasts, bacteria, plants and animals which are among the most important enzymes used in biotechnology. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics (Pandey et al., 2000).

Alpha-amylase enzymes account for about 30 % of the world's enzyme production. Bacteria which can produce the amylase are widely present in nature and can easily be screened and tested for the production of amylase. Commercially used alpha-amylase is obtained mostly from different types of *Bacillus* sp. (Kumar and Duhan, 2011). Amylase enzymes are unstable and lose their property in various conditions like: high temperature, pH, and different chemicals used in the reaction and research is still on-going to find out the bacterial strain which produce more stable amylase enzyme with higher production rate within low cost.

The occurrence of promising starch hydrolyzing amylase from microorganisms enable microorganisms to degrade a variety of agricultural products efficiently (Gashaw and Amare, 1999b).

In spite of these facts, insufficient work was carried out in the production and characterisation of fungal starch degrading amylases from environmental sources. In order to fill some of these gaps of research, the study was aimed to isolate, produce, optimize and characterise the alpha amylase obtained from yeast from different fermented foods.

2. METHODOLOGY

2. 1. Sample collection

Fermented foods and dairy samples such as: palm wine, wara, pito, nunu, ogi, were collected from various markets within Ibadan metropolis. The samples were transported to the laboratory in sterile containers.

2. 2. Isolation of yeasts

One gram of each sample was serially diluted in distilled water and pour plated into Yeast extract agar (YEA) media. The plates were incubated at 30 °C for 3 days. After 3 days incubation colonies with distinct morphological differences such as colour, shape, size were picked and purified by streaking and the yeast isolates were stored in the refrigerator at 4 ºC for future use (Harrigan and MacCance, 1966).

2. 2. 1. Characterization and Identification of Yeast Isolates

The Identification of the isolates was done using standard morphological and physiological test and identification keys according to the method of (Kurtzman et al., 2003; Barnet, 2007). These includes: macroscopic identification, microscopic evaluation and sugar fermentation. The biochemical tests are: Urea Hydrolysis, Citrate Utilization test, Growth in 10% NaCl Growth in 50% Glucose and 5% Glucose, Tolerance of Yeast Isolates in 1% Acetic Acid, Nitrate Assimilation Test and Gelatin liquefaction test (Harrigan and MacCance, 1966).

2. 3. Screening of potent *a*-amylase producing yeast

2. 3. 1. Primary screening

Yeast strains were screened for amylolytic activity by starch hydrolysis test on starch agar plates [Yeast extract agar medium containing 1% (w/v) potato starch at pH = 7.0]. The yeast strains were spot inoculated on the starch agar plates and incubated at 40 °C for 24h. After incubation, a 1% iodine solution [2% (w/v) KI+1% (w/v) I_2] (freshly prepared) was flooded on the starch agar plate. Presence of blue reddish color around the colonies was considered as negative result and a clear zone of hydrolysis surrounding the colonies indicated as positive result. Each colony producing largest clear halo of hydrolysis was considered a potential *a*-amylase producer. Such colony was selected for further investigations.

2. 3. 2. Secondary screening

The selected colonies from above were further screened on Amylase activity medium (AAM) which contain soluble starch 5g/L; peptone 5g/L; yeast extract 5 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; $FeSO_4.7H_2O$ 0.01 g/L; NaCl 0.01 g/L; agar 15g/L plates. Incubation at 30 °C was carried out for 3 days, after which the plates were stained with Lugol solution (Gram's iodine solution: 0.1% I_2 and 1% KI). The colonies forming the largest halo zone were selected for further investigation (Fossi et al., 2009).

2. 4. Amylase production and assay

Extracellular amylases were produced in submerged fermentation; this production was carried out in 250 mL Erlenmeyer flasks containing 100 mL of liquid Amylase activity medium

(AAM) buffered with acetate buffer (pH 5.5). The media were then incubated at 30 °C in an orbital shaker set at 1500 rpm for 48 h. Three replicate fermentations were carried out for each culture medium. The enzyme assay was performed according to the method by Rick and Stegbauer (1974).

2. 5. Inoculum preparation

The strain of yeast was streaked on Yeast extract agar plate and incubated for 24 h at 40 °C. Then, a loopful of freshly grown yeast cells were transferred into100 mL of Yeast Extract liquid medium [tryptone 10; NaCl 10; yeast extract 5 (g/L)] and incubated at 40 °C. Inoculum was taken from the early exponential phase of growth after 24 h.

2. 6. Optimization of physicochemical parameters

2. 6. 1. Effect of Temperature

To study the effect of temperature on amylase production in the submerged fermentation, fermentations were carried out at different temperatures (30, 35, 40 and 50 °C).

2. 6. 2. Effect of pH

The fermentation medium was prepared with varying the pH values (4.0, 5.0, 6.0 and 7.0) to investigate the effect of initial pH on the production of amylase. The media was buffered with acetate buffer (for pH 3-6) and Na-phosphate buffer (for pH 6-7).

2. 6. 3. Effect of Incubation time

The production medium for pectinase and mannanase were harvested at different incubation time (24-72) hrs and assayed to determine the best time that supports enzyme production

2. 6. 4. Effect of nitrogen sources

To investigate the effect of different nitrogen sources on alpha-amylase production, peptone and yeast extract as the only nitrogen sources in the basal medium were replaced with different nitrogen sources such as: urea, casein and ammonium sulphate at 1% (w/v) concentrations.

2. 6. 5. Effect of carbon sources

Similarly, effect of various carbon sources such as cassava flour, corn flour, potato flour, soluble starch and non- gelatinized soluble starch at 1% (w/v) concentrations were examined by replacing starch as the only carbon source in the basal medium.

2. 6. 6. Effect of surfactants

Effect of various surfactants on a-amylase production was also evaluated by adding Sodium dodecyl sulphate (SDS), Dimethyl sulfoxide, polyethylene glycol, Tween80 and Triton X-100 at 2% (w/v) into the finally optimized medium.

2. 7. Protein Determination

For the reaction mixture, test tubes were arranged in duplicates containing 0.0- 1.0 mL of 0.2 mg of protein stock solution (2 mg/ml BSA-Bovine Serum Albumin) and brought up to 1ml with distilled water. For the test mixture, 0.5 ml of sodium acetate buffer pH 5.5 was added to 0.5 ml of the crude enzyme

To both the reaction and test mixture, 5 ml of bovine serum albumin solution was added and the mixture was allowed to stand for 10 mins. 0.5 mL of dilute Folin-ciocalteau reagent was added and then the solution was mixed thoroughly and allowed to stand for 30 mins under room temperature. Protein content of the enzyme was determined by the method of Lowry et al (1951), using Bovine Serum Albumin as standard. The absorbance was read at 750 nm and the protein concentration was determined thereafter.

2. 8. Purification of enzyme

One liter of cell-free supernatant from optimized medium was fractionated into four fractions with ammonium sulfate (0-30%, 30-60%, 60-90% and 90-100%). Precipitated proteins were collected by centrifugation at 10,000×g for 10 min at 4 °C and pellet obtained from each fraction was dissolved in 5 mL of 50 mM Tris-HCl buffer (pH 7.0). Then, resuspended protein precipitates were dialysed overnight against 1 L (three changes) of the same buffer at 4 °C with continuous stirring to completely remove ammonium sulfate. Alpha-amylase activity was measured in each fraction applying DNS method: 0.5 ml of dialysed partially purified enzyme was added to 0.5 mL soluble starch1% (w/v) in 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $CaCl_2$, test tubes were covered and incubated for 5 min at 65 °C in a water bath. Then, 1 mL DNS reagent was added to each tube to stop the reaction and placed in boiling water bath for 5 min, after cooling the samples in a cold water bath, the absorbance was read at 540 nm. The dialysed enzyme was loaded on the gel permeation column (Sephadex S-100). All the eluted fractions were estimated for enzyme activity and absorbance was read at 540 nm and 575 nm respectively. The fractions showing the highest enzyme(s) activity were pooled and assayed for protein content. The specific activity of purified enzyme fractions was compared to that of crude enzyme and fold purification was calculated.

2. 8. 1. Characterization of partially purified enzyme

2. 8. 2. Effect of pH on *a*-amylase activity and stability

The effect of pH on ***a*** -amylase activity of partially purified enzyme was evaluated by performing the alpha-amylase assay in the following buffer systems: 50 mM sodium acetate (pH 3.0-5.0), 50 mM potassium phosphate (pH 6.0–7.0), 50 mM Tris-HCl (pH 8.0–9.0) and 50 mM glycine-NaOH (pH 9.0–11.0). To study the influence of pH on the stability of alpha-amylase, partially purified enzyme was mixed with the selected buffer systems at a ratio of 1:2 and incubated at 30 °C for 30 min, and then the residual activity (%) was determined under standard assay conditions.

2. 8. 3. Effect of Optimum temperature on *a*-amylase activity and stability

To determine the optimum temperature, activity of the partially purified enzyme was measured at temperatures ranging from 30 °C to 50 °C for 30 min at 50 mM Tris-HCl buffer (pH 7.0. The residual activity was determined under standard assay conditions.

2. 8. 4. Effect of Substrate Concentration on *a*-amylase activity.

The effect of substrate concentration on the activity of alpha-amylase was determined by incubating the enzyme with 0.5 to 2.0 mg/mL starch at an interval of 0.5 using the buffer at the pH with highest activity and the temperature at which highest activity was determined.

2. 8. 5. Effect of metal salts

The effect of metal salts on α-amylase production is determined by adding different metal salts in the fermentation medium. The metal salts selected for present study are $FeCl_2$, $MgSO_4$, $CaCl_2$ and $CuSO_4$ at 0.1% concentration.

2. 8. 6. Thermo stability of amylase

The thermal stability of the enzyme was determined by incubating enzyme fractions at various temperatures between 25 ºC to 60 ºC without the substrate for 1h at 10 min intervals, aliquots of 0.5 ml of the incubated enzyme were assayed for activity.

2. 9. Molecular Characterization of the yeast isolates

2. 9. 1. DNA Extraction Protocol

The extraction of the yeast genomic DNA for molecular analysis was carried out according to the method of Arnold *et al* (2011).One hundred (100) mg of fungal mycelia was put into a sterile mortal and 1ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05 mg/ml) was added and macerated with sterile pestle, the extract was transferred into 1.5 ml Eppendorf tube and 50 µl of 20% Sodium Dodecyl Sulphate (SDS) was added and incubated in a waterbath at 65 °C for 30 minutes.

2. 9. 2. PCR Analysis

PCR sequencing preparation mixture consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each of the ITS 1 and ITS 4 primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles consisting of 94 °C for 30 s, 30 secs annealing of primer at 55 °C and 72 °C for 1 minute 30 second and a final termination at 72 °C for 10 mins and chilled at 4 °C.

2. 9. 3. Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

2. 10. Statistical Analyses

The results obtained in this study were subjected to statistical analyses.

3. RESULTS

3. 1. Yeast isolates obtained

Out of the forty- three (43) yeast isolates obtained from the fermented foods. The occurrence of yeast isolates obtained from the fermented food shows that the predominant organisms in the fermented foods are: *Candida* sp. *Geotrichum* sp, *Saccharomyces* sp, *Pichia* sp and *Kluyveromyces* sp. as shown in Figure 1. Ten (10) yeast isolates listed in Table 1 were shown to possess alpha amylase activity after being screened. Yeast isolates WR4 and OG9 had the largest zone of starch hydrolysis (24.5 mm and 22 mm respectively) and thus were selected for this research. The result of the biochemical identification of the ten (10) isolates positive for alpha amylase activity is shown in Table 2. Molecular characterization of the two yeast isolates showed 99% identity similar to *Candida tropicalis* strain IFM 63517 (OG9) and *Candida tropicalis* strain CMC 1836 (WR4) internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene partial sequence.

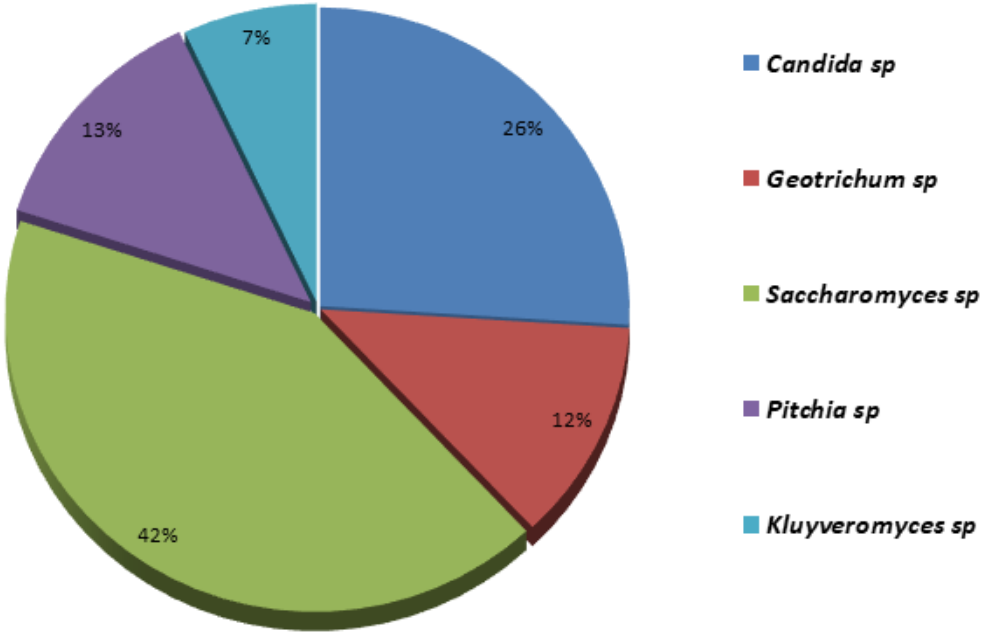

Figure 1. Occurrence of yeast isolates obtained from fermented food

Table 1. Isolates Positive for Primary Amylase Screening

ISOLATES/CODES	ZONE DIAMETER [mm]
WR5	**10.4**
OG9	**22.0**

KN3	**9.0**
YU3	**16.0**
PW8	**9.5**
WR4	**24.5**
PW5	**14.0**
KN6	**11.4**
KN6	**12.0**
WR9	**8.6**

Table 2. Secondary screening showing amylase activity of yeast isolates obtained from fermented foods.

Isolates	**ENZYME ACTIVITY (U/ml)**
WR5	*32.22 ± 0.11
OG9	44.45 ± 0.35
KN3	12.63 ± 0.22
YOU3	35.63 ± 0.32
PW8	14.45 ± 0.15
WR4	52.46 ± 0.02
PW5	22.86 ± 0.01
OG2	18.28±0.14
KN6	21.23±0.03
WR9	11.65±0.04

3. 2. Optimization of fermentation parameters.

3. 2. 1. Optimization of incubation period.

The yeast isolates tested for amylase production was subjected to incubation period of 24-48 hours. Amylase activity for both yeast isolates were both highest at 48 hours incubation. *Candida tropicalis* IFM63517 shows a activity of 62.8 u/mL while *C. tropicalis* CMC1836

shows amylase activity of 44 u/mL at 48h incubation. The least enzyme activity was recorded at 72 hours which is 21.8 u/ml and 8.1u/mL for both *C .tropicalis* IFM 63517 and *C. tropicalis* CMC 1836 respectively (Figure 2).

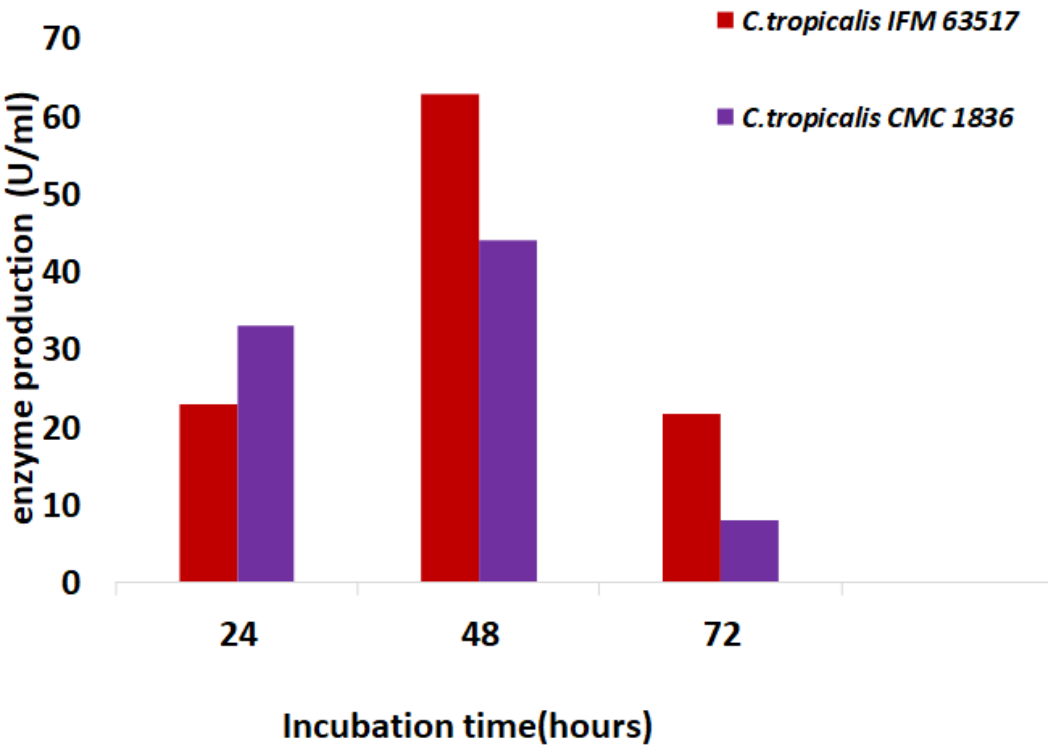

Figure 2. Effect of incubation time on production of α-amylase by the yeast isolates obtained from fermented foods.

3 .2. 2. Optimization of temperature

Temperature is one of the important factors that influence the production of enzymes. Temperature range of 30 °C – 45 °C was observed in this study as seen in Figure 3. Production of amylase by *C.tropicalis* IFM 63517 is found to be optimum at 30 °C with amylase activity at 62.3 u/mL while the optimum production temperature for *C. tropicalis* CMC 1836 is 35 °C with amylase activity of 50.1 u/mL. The least temperature that supported amylase production was 45 °C with amylase activity 14.7 u/mL and 8.0 u/mL for both *C. tropicalis* IFM 63517 and *C.tropicalis* CMC 1836 respectively.

3. 2. 3. Optimization of pH

pH is the power of the hydrogen ion and it is one of the most importance factor in enzyme production. pH range of 4-7 was observed in this study and Amylase production in *C. tropicalis* IFM63517 increases as pH increases from 4 (31.6 u/mL) reaching a maximum of 5 (67.8 u/mL) above pH of 5 there was a reduction in amylase production reaching the barest minimum at pH 7 (25.1 u/mL). The maximum activity for *C. tropicalis* CMC 1836 was observed at pH 5 (58.4

u/mL) this is said to be the optimum. While the lowest activity for *C. tropicalis* CMC 1836 was observed at pH 7 (30 u/mL) as seen in Figure 4.

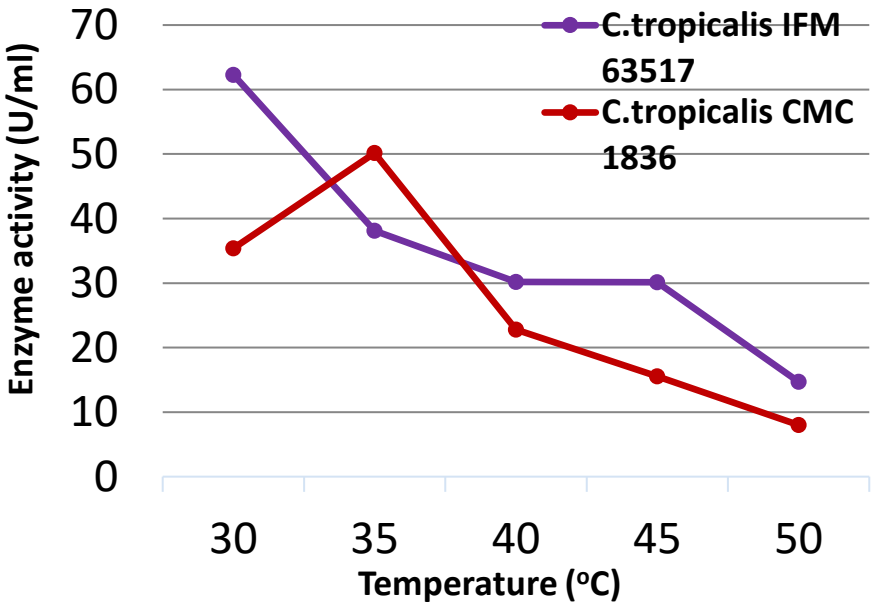

Figure 3. Effect of temperature on production of α-amylase by the yeast isolates obtained from fermented foods.

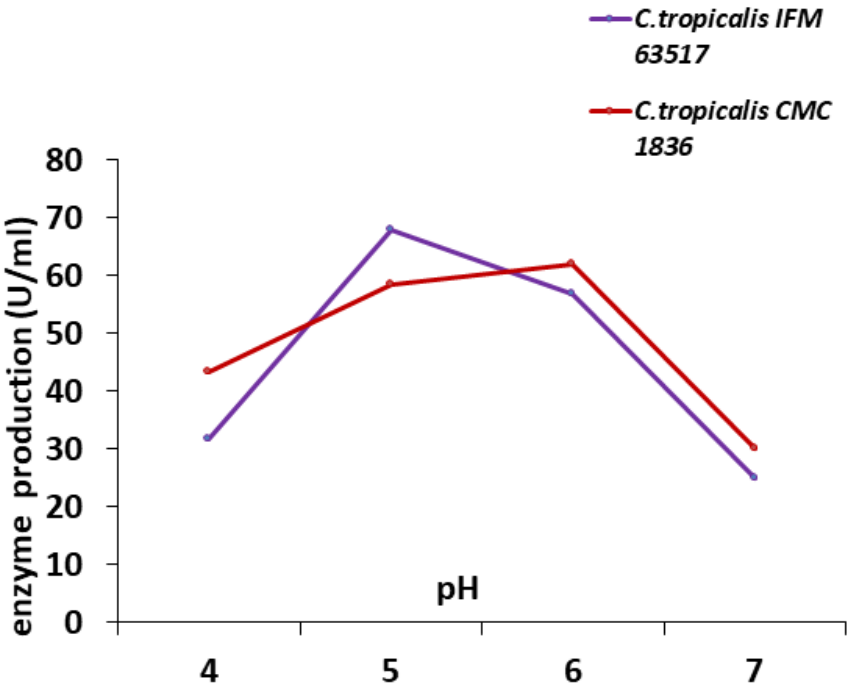

Figure 4. Effect of pH on production of α-amylase by the yeast isolates obtained from different fermented foods.

3. 2. 4. Optimization of carbon sources

The effect of various carbon sources was studied with additives such as soluble starch, cassava flour, corn flower and potato flour. Soluble starch serves as the control substrates for the production of amylase in this study. It is apparent from (Figure 5) that soluble starch supports the highest production of amylase in both *C. tropicalis* IFM63517 and *C. tropicalis* CMC 1836 with enzyme activity being 66.4 u/mL and 72.9 u/mL respectively. The second highest production of amylase for *C. tropicalis* CMC 1836 was observed in potato flour with enzyme activity at 62.7 u/mL while *C. tropicalis* IFM 63517 produced a considerable amount of amylase using corn flower with enzyme activity at 46.2 u/mL. The additives that showed the least amount of produced enzyme in *C. tropicalis* IFM 63517 was observed to be potato flour at 39.6 u/mL, while the least production for *C. tropicalis* CMC 1836 was observed in cassava flour at 39.1 u/mL enzyme activity.

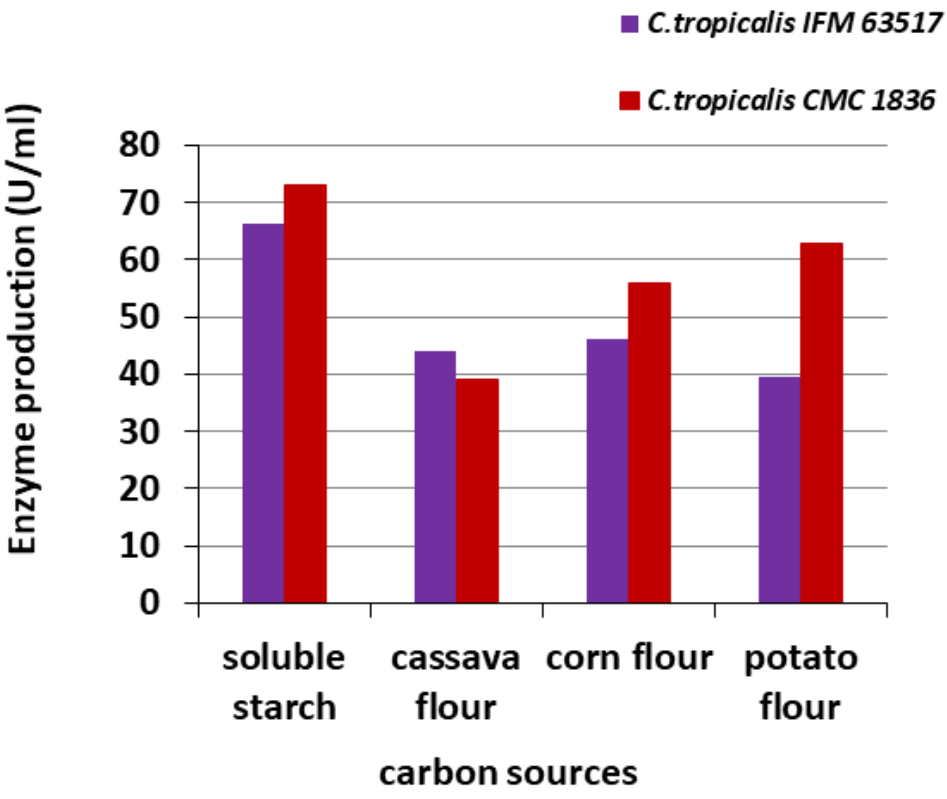

Figure 5. Effect of carbon sources on production of α-amylase by the yeast isolates obtained from different fermented foods.

3. 2. 5. Optimization of Nitrogen sources

The effect of different nitrogen sources as additives in the production of amylases. Additives such as casein, yeast extract, urea, ammonium sulphate and peptone was observed in this study. Yeast extract supports the maximum amylase production for both *C. tropicalis* IFM 63517 and *C. tropicalis* CMC 1836 at 71.8 u/mL and 67.8 u/mL respectively (Figure 6). The

second highest activity for *C. tropicalis* IFM 63517 was observed in casein at 54.5 u/mL while that of *Candida tropicalis* was observed to be peptone at 45.0 u/mL. Ammonium sulphate gave the least production of amylase in *C. tropicalis* IFM 63517 with enzyme activity at 25.3 u/mL, while the least production of amylase in *C. tropicalis* CMC 1836 with enzyme activity 25.1 u/mL was observed in urea.

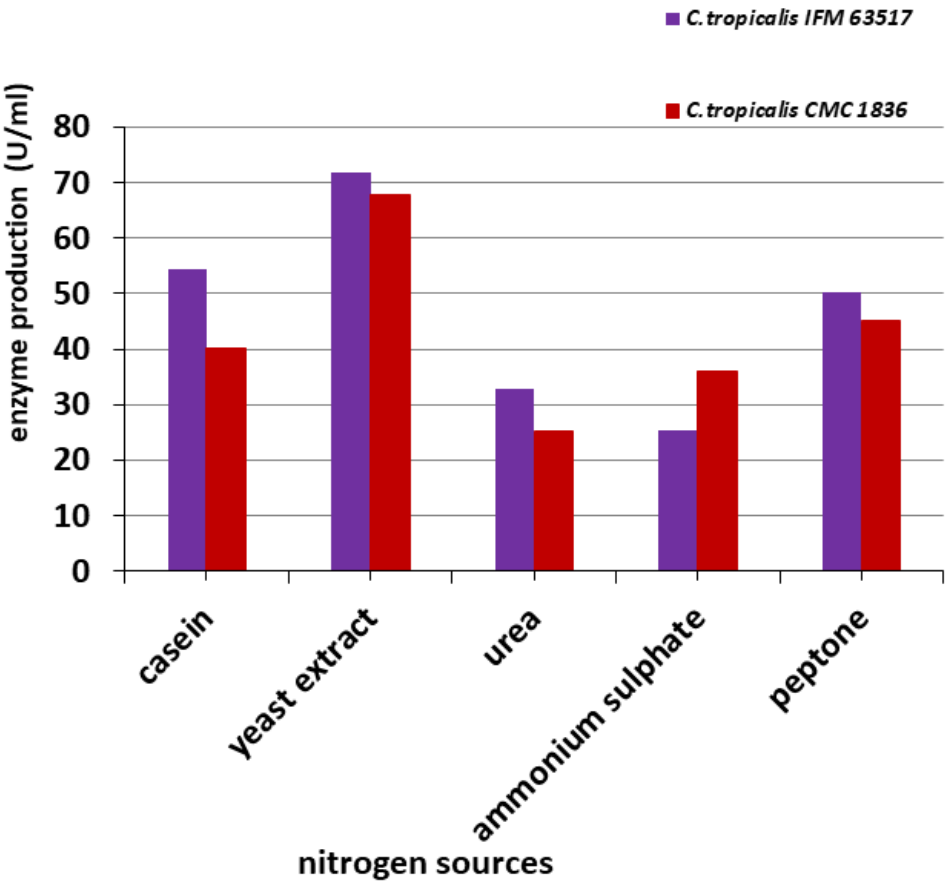

Figure 6. Effect of nitrogen sources on production of amylase by the yeast isolates obtained from different fermented foods.

3. 2. 6. Optimization using surfactants

Surfactants has been observed to stimulates the production of enzymes by reducing the surface between the cell walls of organisms and the growth media, thereby, creating pores in the cell wall allowing free flow of nutrients into the organism.

In this study, surfactants used were sodium dodecyl sulphate (SDS), dimethyl sulfoxide (DMSO), polyethylene glycol, Tween-80, Triton X-100. Tween-80 supports the maximum enzyme production in both *C. tropicalis* IFM 63517 and *C. tropicalis* CMC 1836 with enzyme activity of 43.4 u/mL and 68.2 u/mL respectively. Triton X-100 was the second highest producer in both isolates, while polyethylene glycol produced the least amount of amylase in *C. tropicalis* IFM 63517 and *C. tropicalis* CMC 1836 with enzyme activity at 21.1 u/mL and 12 u/mL respectively as seen in Figure 7.

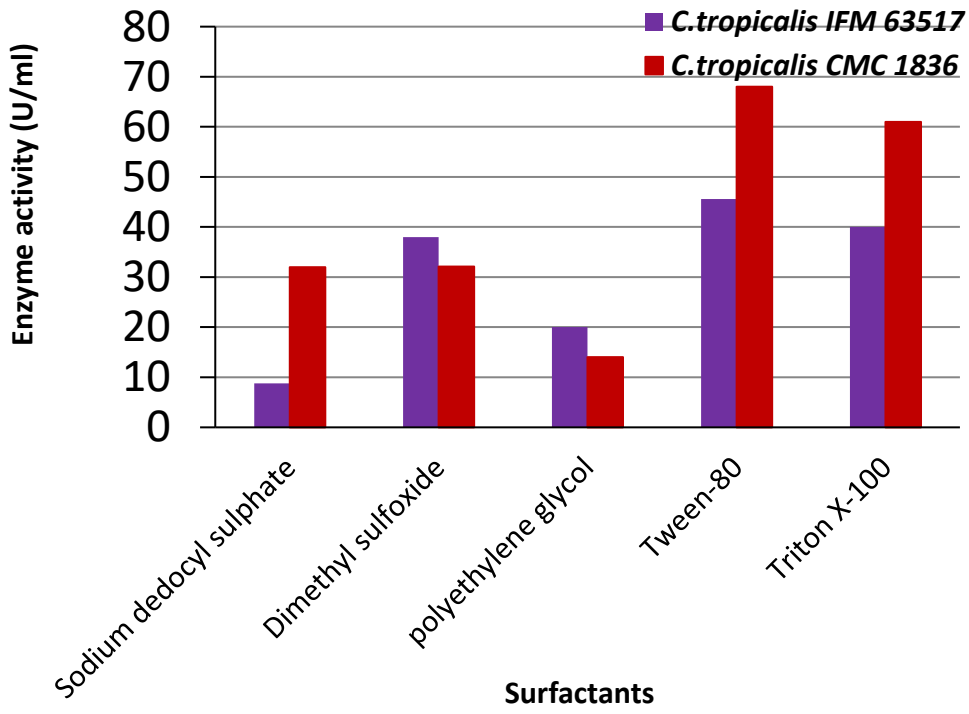

Figure 7. Effect of different surfactants on production of amylase by the yeast isolates obtained from different fermented foods

3. 3. Characterization of crude Amylase

3. 3. 1. Optimum temperature for amylase activity

The effect of temperature on the activity of enzyme was examined by determination of the activity at various temperature ranges. Results obtained indicated that the optimum temperature for both *C. tropicalis* IFM63517 and and *C. tropicalis* CMC 1836 was 35 °C (68.0 u/ml and 59.0 u/ml respectively) as shown in Figure 8.

3. 3. 2. Optimum pH for Activity of crude Amylase

The effect of pH on the activity of enzyme was examined by determining the activity at various pH ranges (4-7). Results obtained indicated that the enzyme activity was optimally active at pH 6.0 (60.1 U/ml and 56.2 U/ml respectively) for *C. tropicalis* IFM 63517 and *C. tropicalis* CMC 1836 (Figure 9).

3. 3. 3. Optimum Temperature for the stability of crude Amylase

The effect of temperature on the amylase stability was determined by sustaining the enzyme at various temperatures ranging from 40 °C – 90 °C for 30 min. Highest amylase stability was observed at 40 °C for both *C. tropicalis* IFM 63517 and *C. tropicalis* CMC 1836 (66.0 U/mL and 70.3 U/mL). The stability of amylase for both isolates decreases as temperature decreases reaching the barest minimum at 90 °C. 21.2 U/mL and 25.7 U/mL for both *C. tropicalis* IFM 63517 and *C.tropicalis* CMC 1836 respectively (Figure 10).

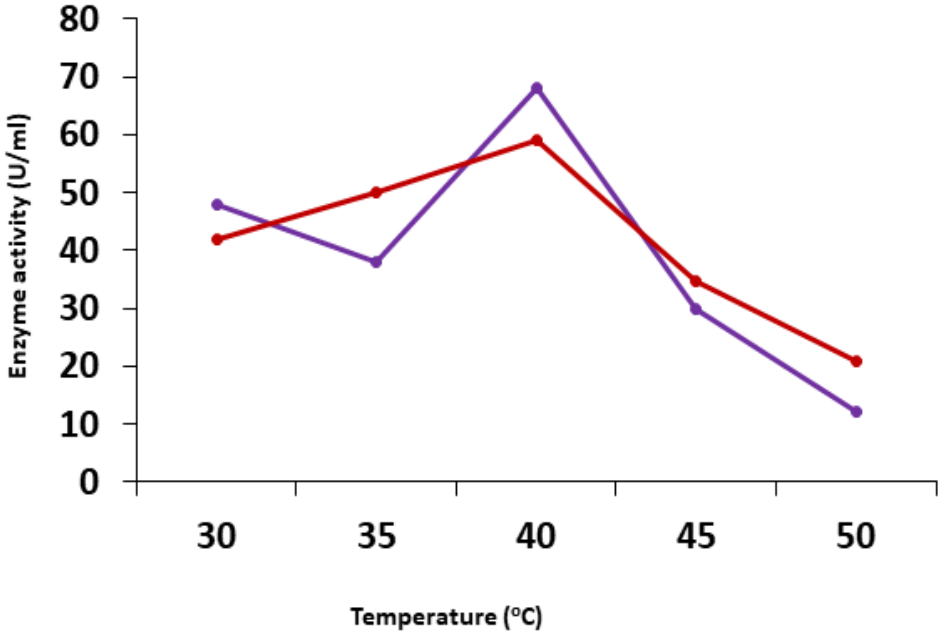

Figure 8. Effect of temperature on activity of α-amylase by the yeast isolates obtained from different fermented foods.

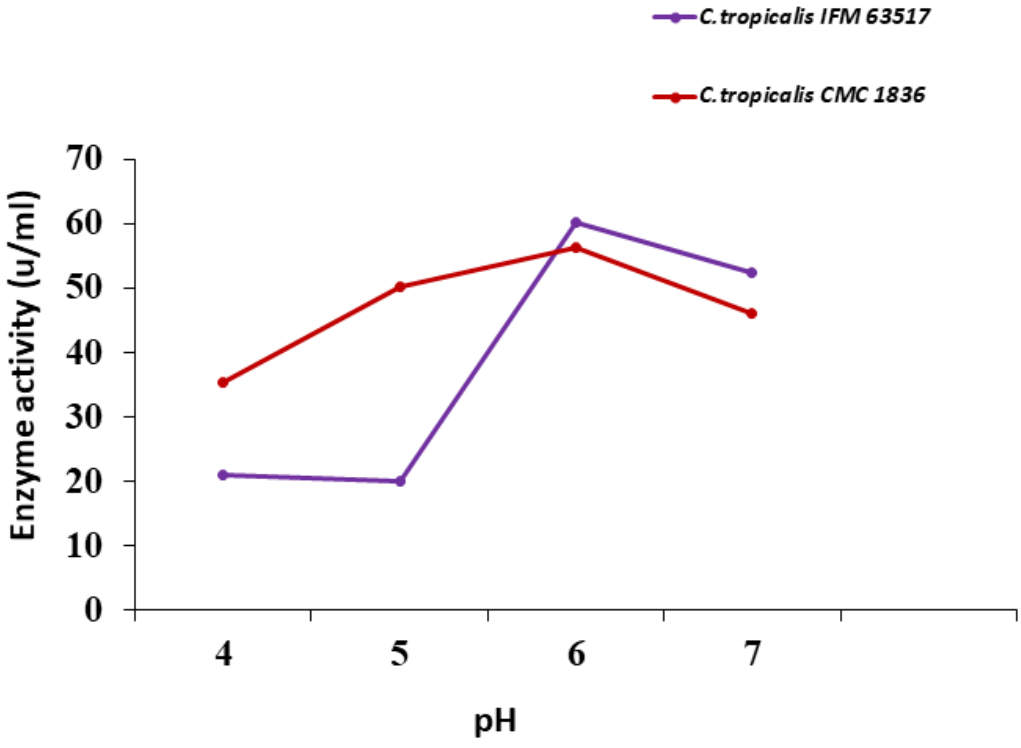

Figure 9. Effect of pH on activity of α-amylase by the yeast isolates obtained from different fermented foods.

3. 3. 4. Optimum Temperature for the stability of crude Amylase

The effect of temperature on the amylase stability was determined by sustaining the enzyme at various temperatures ranging from 40 °C – 90 °C for 30 min. Highest amylase stability was observed at 40 °C for both *C.tropicalis IFM 63517* and *C. tropicalis* CMC 1836 (66.0 U/mL and 70.3 U/mL). The stability of amylase for both isolates decreases as temperature decreases reaching the barest minimum at 90 °C. 21.2 U/mL and 25.7 U/mL for both *C. tropicalis* IFM 63517 and *C. tropicalis* CMC 1836 respectively. (Figure 10).

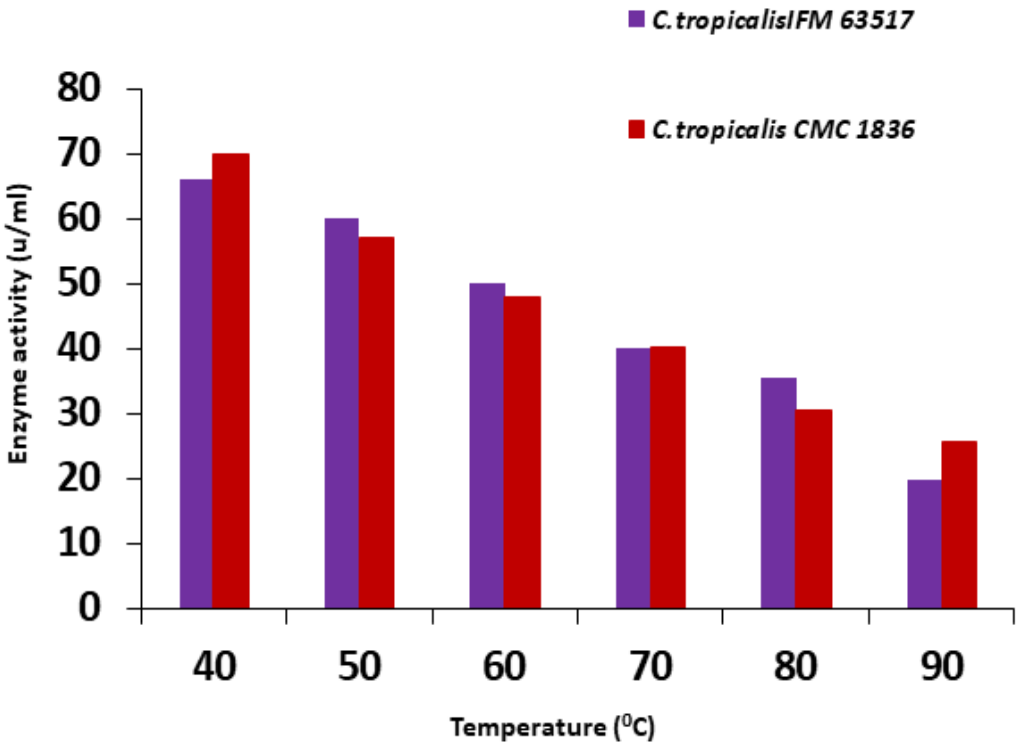

Figure 10. Stability of α-Amylase enzymes at different temperatures

3. 3. 5. Optimum substrate concentration for crude amylase activity

The effect of substrate concentration on the activity of enzyme was examined by determining the activity at various concentrations (0.5, 1, 1.5,2 and 2.5% w/v). Results obtained indicated that the enzyme activity was optimally active at 1% w/v (74.2 U/mL and 71.0 U/mL respectively) for both *C. tropicalis* IFM 63517 and *C. tropicalis* CMC 1836 (Figure 11).

3. 3. 6. Effect of metal ions on crude amylase activity

The effect of various metal ions ($CaCl_2$, $FeCl_2$, $MgSoO_4$, and $CuSO_4$) on activity of enzyme was examined (Figure 12). Highest enzyme activity was observed in $CaCl_2$ for both *C. tropicalis* IFM 63517 and *C. tropicalis* CMC 1836, while lowest activity was observed in $CuSO_4$ in both yeasts.

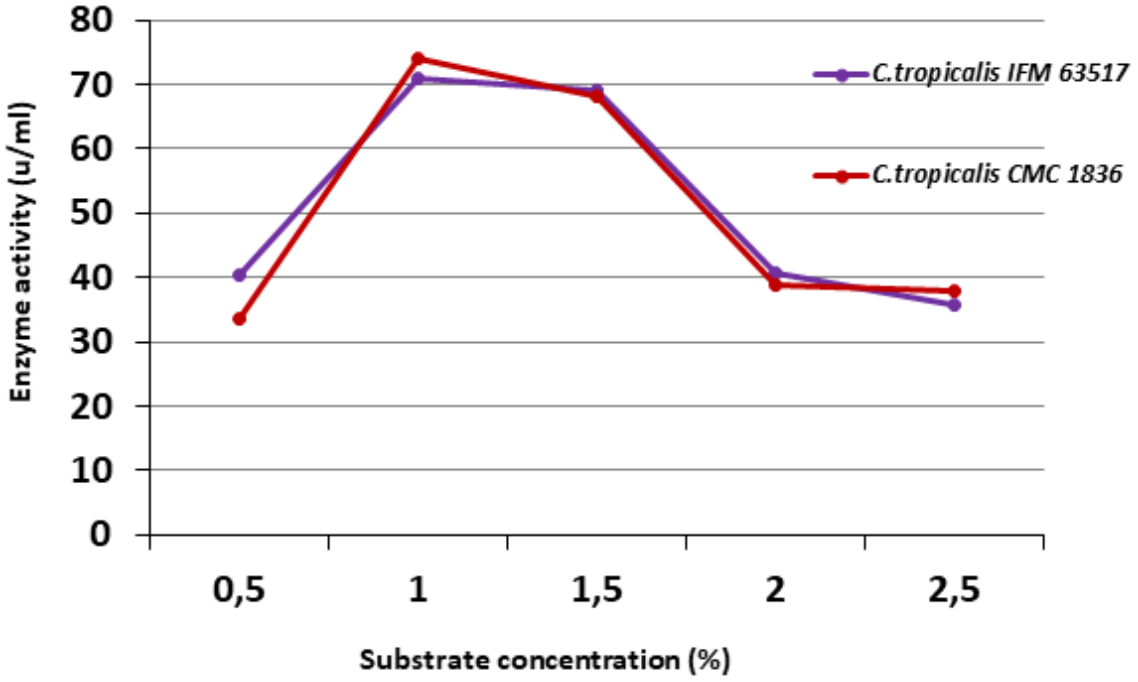

Figure 11. Effect of substrate concentration on the activity of α-amylase from the yeast isolates obtained from different fermented foods.

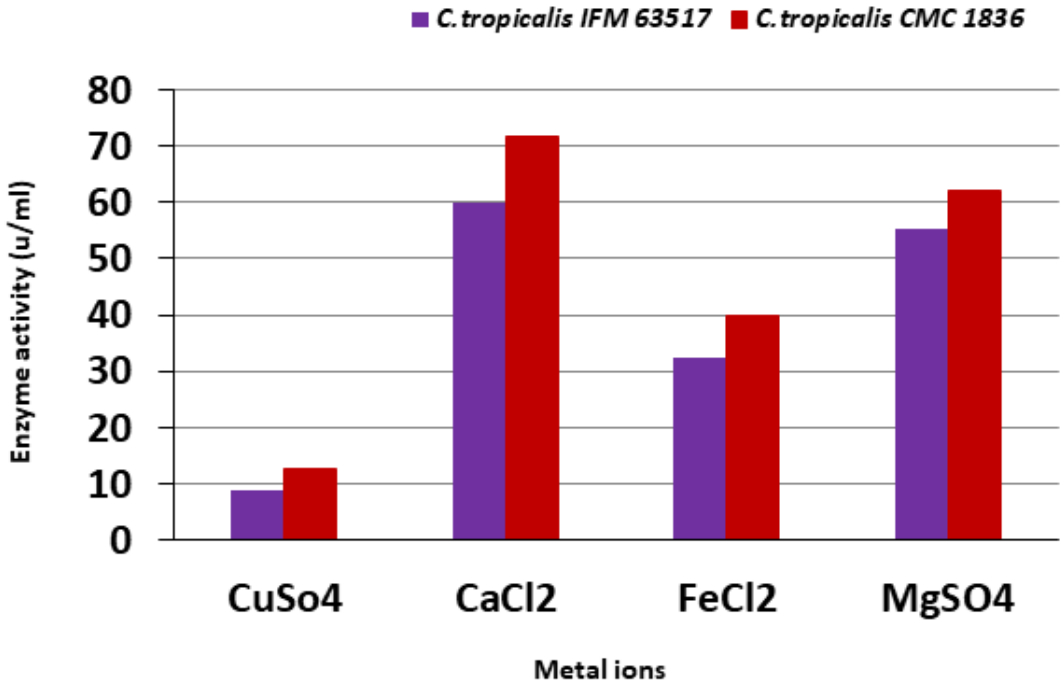

Figure 12. Effect of various metal ions on the activity of αamylase the yeast isolates obtained from fermented foods.

3. 4. Purification of crude Amylase

The supernatant of *C. tropicalis* IFM 63517 with enzyme activity of 3900 U/50ML and specific activity of 30 U/mL were used as crude enzyme solution and subjected to partial purification by ammonium sulfate precipitation in two fractions of 0%-50% and 50%-80% saturation. The specific activity of precipitated *C. tropicalis a* -amylase was 56.85 U/mg with 1.89 purification fold respectively. By gel filtration chromatography, their specific activity was 120.83 U/mL with purification fold of 4.02 (Table 3).

Table 3. Summary of the purification of alpha amylase from *C.tropicalis* strain IFM 635

Purification Steps	Total volume	Total enzyme activity (U)	Total protein content (mg)	Specific activity (U/mg)	Purification fold	% yield
Crude cellulase	50	3900	130	30	1	100
Ammonium sulphate precipitation	50	2956	52	56.85	1.89	75.79
Dialysis	45	2050	29	70.68	2.35	52.56
Gel filtration	10	1450	12	120.8	4.02	37.17

4. DISCUSSION

In this study, forty- three (43) yeast isolates were obtained from different fermented foods and dairy products. After preliminary screening of the forty- three (43) isolates for their alpha amylase producing ability, ten (10) which represented 23% of the total were positive for alpha amylase production. This was similar to the report of Pandey et al (2000) and Chi et al (2009) that a number of yeast isolates such as *Saccharomyces* sp., *Kluyveromyces* sp., *Candida* sp. etc. isolated from fermented foods possess inherent ability to produce alpha amylase.

According to Banwo et al (2015) the ready availability of yeast in such food products is as a result of the high sugar content hence leading to the addition of desirable flavour to fermented foods. Various genera of yeast including: *Saccharomyces, Candida, Geotrichum* and *Kluyveromyces* were isolated in the course of this work and these genera are in the same range with that obtained by Banwo et al (2015) who isolated *Saccharomyces, Candida, Geotrichum, Pichia* and *Kluyveromyces* from ogi and pito.

This best two producers (*Candida tropicalis* IFM 63517 and *Candida tropicalis* CMC 1836) of alpha amylase were studied under various optimized condition for their producing ability. Incubation time, temperature, carbon sources, nitrogen sources, effect of surfactants and pH are all important factors that affect extracellular alpha amylase production. Substrate(s) should provide all needed nutrients to the microorganism for its growth. The substrate used for the production of extracellular alpha amylase was soluble starch.

Also, in this research, submerged fermentation technique was applied due to the fact that temperature, pH, moisture, substrate concentration during cultivation would have been difficult to control under limited water availability (Holker et al., 2004).

In this study, total amylase activities in the liquid media after incubation at 30 °C was achieved at 48hours for both isolates. This agreed with the work of Souza (2010) who reported 48 hours as the best incubation time for maximum alpha amylase production.. Increase in the incubation period resulted into the decrease in the production of alpha amylase. This may be due to the fact that after maximum production of alpha amylase (maximum incubation time), the production of other by-products increases hereby leading to a depletion of nutrients. This is in accordance with the work of Haq et al., (2003) who reported that the by- products produced during incubation period inhibited the growth of yeast and hence depletion in enzyme production.

The optimum temperature for alpha amylase production in both isolates was between 30 °C - 35 °C. This result agreed with the work of Gupta et al., (2003) who reported similar temperature for alpha amylase production in *Candida tropicalis.* Similar results were also reported by Rene and Hubert (1985) with *Filobasidium capsuligenum*.

The effect of pH on the production of alpha amylase in this study revealed that amylase production by the isolates was at their maximum between pH 4 – 6. This is in accordance with the work of Moreira et al. (1999) who found that amylase are produced maximally by fungal organisms in an acidic medium. Also, this result is in agreement with the work of Hostinova (2002), who found that the optimal pH for glucoamylase and alpha -amylase in *Candida tropicalis* was 5.0-6.2. Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in the enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium (Gupta et al., 2003). Amylase is a pH sensitive enzyme and the selection of optimal pH is essential for the production of alpha -amylase (Mcmohan, 1999).

The study of the effect of carbon source showed that the best substrate for the production of alpha- amylase by the two isolates was soluble starch. This report is in support of the report by Fabiana et al., 2008.that the best carbon source for alpha -amylase production is soluble starch

The effect of nitrogen sources on the production of alpha -amylase under submerged fermentation showed that yeast extract was found to be a better nitrogen source for both isolates. This is in agreement with the work of Arneson et al., (1998) who reported that yeast extract is the best nitrogen source for amylase production probably due to its high content in minerals, vitamins, coenzymes and nitrogen components. Pederson (2000) also reported that amylase production by *C. tropicalis* was greatly influenced by organic nitrogen sources especially yeast extract and casein. However, this was in contrast with the work of Hoa and Hung, (2013) who reported urea as the best nitrogen source. The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The nature of the compound and the concentration that we used might stimulate or down modulate the production of enzymes (Arneson et al., 1998

The effect of various surfactants on the production of alpha -amylase was carried out on both isolates. From the result obtained in this study, Tween-80 produced the highest amount of alpha- amylase enzyme. This is in agreement with the work of Balkan and Ertan (2007). However, this result does not support the work done by Arneson et al., (1998) in which Triton X-100 supported the highest enzyme production. Surfactant in fermentation medium has been

known to increase enzyme production by increasing membrane permeability of the cell thereby allowing the influx of nutrient into the cell hence the increase in enzyme production (Arnesen et al., 1998). According to the work of Reese and Anne (2008) who worked on the effect of different surfactant as stimulants in the production of alpha -amylase in *Saccharomyces cerevisiae*. They found out that Tween-80 had an effect on enzyme production where the microorganism was stimulated thereby leading to an increase in enzyme production.

In this study, the effect of temperature on amylase enzyme activity revealed that 40 °C was optimum and at the tested higher temperatures, the enzyme production decreased which might be due to growth reduction and enzyme inactivation or suppression of cell viability (Srivastava et al., 1986). A similar result was reported by Prabhakaran (2009). In contrast, low temperature values may reduce the metabolism of the microorganism and consequently, the enzyme synthesis.

The partially purified alpha -amylase was subjected to different substrate concentration and it was found out that enzyme activity in both *Candida tropicalis* IFM 63517 and *C. tropicalis* CMC1836 was highest at 1% concentration this is in agreement with the work of Hostinova (2002) who observed that the optimum substrate concentration for amylolytic enzymes produced by *S. fibuligera* was 1%. This could be because the enzyme has a low maximum kinetic energy (K_m) which consequently requires a low substrate concentration to become saturated (Hostiniva, 2002).

In this study, the effect of metal ions on amylase activity was studied. it was observed that highest enzyme activity was found in Ca^{2+} from the crude enzyme obtained from both isolates and this is in agreement with the work of Pandey et al., (2000) who reported that most of amylases are known to be metal ion-dependent enzymes and that calcium ions provide a binding link between enzyme and substrate holding the substrate and the active site of the enzyme thereby stimulating enzyme production. Pandey et al., (2000) also reported that amylases are known to be a Ca^{2+} metalloenzyme.

During purification of the enzymes, after the protein was precipitated, dialysis was carried out to remove excess salt. It also changes the buffer composition of solutions of biomolecules too large to pass through the membrane (Rosenberg, 2004). Therefore, it was observed that there was a decrease in volume of about 5mL of the enzyme after dialysis. This may be due to the further separation that occurred between the low and high molecular weight proteins that were initially present (Rosenberg, 2004).

There was significant increase in the activity of pectinase and mannanase after precipitation and subsequently after dialysis. This agrees with the suggestion of Prabhakaran (2009) who suggested that increase in enzyme activity may have occurred due to the removal of impurities (such as other proteins) during dialysis. The specific activity of the crude, precipitated and dialyzed mannanase increased. This also showed an increase after each purification step as, which was in agreement with the suggestion of Prabhakaran. (2009) that for a purification procedure to be successful, the specific activity of the desired enzyme must be greater after the purification procedure than as it was before.

5. CONCLUSION

This study has shown that yeast especially *Candida tropicalis* are viable source of alpha-amylase when supplied with adequate nutritional and cultural conditions due to their production

capability in a wide range of pH and temperatures. It should be noted that alpha- amylase producer strains with increasing enzyme activities at high temperature are promising candidates for industrial application. Soluble starch is a good substrate for alpha- amylase production under submerged fermentation. The optimum incubation period, temperature and pH for alpha - amylase production from the *Candida tropicalis* isolates were 48 hours, 30 °C and 5.5, respectively. Soluble starch served as the best carbon source for enzyme while yeast extract serves as the best nitrogen source for enzyme production.

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