

IMPROVED AMYLOLYTIC ABILITY OF *LACTOBACILLUS PLANTARUM* AND *LACTOBACILLUS BREVIS* STRAINS ISOLATED FROM CASSAVA MASH AND OGI

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ABSTRACT

Lactic acid bacteria were investigated for their ability to degrade starch under various conditions. Three isolates were selected and they include two *Lactobacillus plantarum* strains and one *Lactobacillus brevis* (LP1, LP2 and LB respectively). Growth and amylase production were optimised with different carbon and nitrogen sources, pH, temperature, metal ions and starch concentrations. Obtained data were analyzed with the analysis of variance (ANOVA) using SPSS 2007. All the isolates gave the maximum values for growth and enzyme production at 30 °C while the optimum pH for growth was 5.0 for isolate LP2 and 6.0 for LB and LP1. The best pH for amylase production for the isolates was 6.0. Optimum amylase activity occurred at 50 °C for LP1 and LP2 and at 60°C for LB. All the strains utilised all carbon sources for growth and amylase production. Concentrations of soluble starch affected amylase production considerably. Metal ions at low concentrations increased the activity of the enzyme while micromolar concentrations of EDTA and 2,4 DNP repressed the activity of the enzyme. LP1 and LP2 had the same V_{max} and K_{max} values of 5.0 while LB had the lowest values of V_{max} and K_{max} . LP1 had the highest K_m value.

Keywords: *Lactobacillus plantarum*, *Lactobacillus brevis*, amylase, carbon source, optimised, 2,4 DNP.

INTRODUCTION

Amylase enzymes are applied in industries where sugar forms the basic raw material (Sanni *et al.*, 1992). They are used to break down starches to sugars. Apart from baking and brewing, pharmaceutical industries also make use of amylase extensively. The production of glucose and fructose syrup is mediated by alpha amylase (Sobia *et al.*, 2023).

Different perceptions have been used in studying amylase production. A lot of work has been done on amylase production by mesophilic and thermophilic bacteria (Adetunji and Adejumo, 2018; Asad *et al.*, 2014; Marwan *et al.*, 2020). The production of amylase has been confirmed in many bacteria including lactic acid bacteria (Awasthi *et al.*, 2018; Marwan *et al.*, 2020). Likewise, various types of amylase production had been reported in fungi (Okunwaye *et al.*, 2021). Because starch is the medium for cultivation of amylase-producing microbes, commercial soluble starch is often used as the carbon source. The methods of extraction and purification in large scale production of microbial enzymes form the major steps, and the degree to which the enzyme is purified depends on both economic factors and the intended application (Abdulaal, 2018).

A lot of studies have been carried out on the fermentation of cassava and ogi. These have centred on cyanide removal (Iasmine

et al., 2016), the microorganisms involved in the fermentation process (Andri, 2017), and optimisation of the fermentation process (Andri, 2017). In the fermentation of cassava, a number of microorganisms have been implicated. They include *Corynebacterium* sp., *Geotricum* sp., *Lactobacillus* sp. and *Candida* sp. The majority of lactic acid bacteria isolated during cassava and ogi fermentation belong to the *Lactobacillus plantarum* group (Olaoluwa *et al.*, 2013).

Owing to the abundance distribution of lactic acid bacteria, which have been considered to be safe, in starch materials and importance of amylase in many industries. The production of amylase enzymes by these organisms needs to be investigated. The objectives of this work were to screen for *Lactobacillus plantarum* and *Lactobacillus brevis* isolates with ability to degrade starch, determine the extent of production of amylolytic enzymes in such isolates, and determine the effect of various factors on the activity of the amylase produced.

MATERIALS AND METHODS

Sample Collection and Isolation of Microorganisms from the Samples

Twenty-four (24) samples of cassava mash and ogi used in this study were collected from different markets in Ife, Ikire, Ede and Ogbomoso towns. One (1) g of each of the samples was mixed thoroughly with 9 mL of sterile distilled water, serially diluted up to 10^{-6} . From the final dilution, 0.1 mL was used to inoculate sterile plates of DeMan, Rogosa and Sharpe agar (MRSA) in which the glucose was replaced with soluble starch. The plates were anaerobically incubated in an anaerobic jar containing a gas pack content which reacted to produce hydrogen gas, which created the anaerobic condition in the jar. All plates were incubated at 30 °C for 48 h (Rhaïem *et al.*, 2016).

Screening for Amylase-Producing Lactic Acid Bacteria

Lactic Acid Bacteria were isolated from the samples on DeMan, Rogosa and Sharpe agar having glucose replaced with 2 % soluble starch. Amylolytic activities of the isolates were detected by flooding the plates with iodine solution (a solution of 1 g iodine crystal and 2 g potassium iodide KI in 300 mL distilled water). All the amylase-positive isolates were Gram-stained and tested for catalase production. All the Gram-positive rods and catalase-negative organisms were purified using MRS agar. They were then maintained on MRS agar slant with the same composition at 4 °C before further tests were carried out on them. Identification of the isolates was based on the Gram-reaction and biochemical tests with reference to ABIS (Fazal *et al.*, 2022).

Amylase Assay

Amylase production and activity by the *Lactobacillus plantarum* strains were determined using DNSA reagent method of Saito (1973), as modified by Wood (1965). One tenth millilitre (0.1 mL) of culture supernatant was added to 0.8 mL of a solution containing 1.2 mg of soluble starch in 0.1mL phosphate buffer, pH 6.0. The reaction was stopped by the addition of 0.1mL of 5M NaOH. The enzyme-starch mixture was incubated at 30 °C for 10 min. The amount of sugar thus produced was estimated according to Saito (1973) with 3,5-dinitrosalicylic acid. The absorbance was measured at 540nm on a Jenway 6105 uv/vis spectrophotometer. A standard curve was prepared using standard aqueous maltose solutions of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mg /mL. The calibration so established was used to convert the spectrometer values to maltose equivalents. One unit of enzyme is defined as that amount of enzyme which released 0.1mL of reducing sugar per 0.1mL of culture supernatant (as maltose equivalent) under the stated assay conditions.

Effect of Different Carbon Sources on Growth and Amylase Production by *Lactobacillus planetarium*:

Twenty (20) mL of the prepared MRS medium in which glucose was replaced with equimolar amounts of different sugars (lactose, mannitol, maltose, and galactose) was dispensed into 100ml Erlenmeyer flasks, sterilized, and uninoculated with 2 mL of a 24 h culture of the organism. Growth measurement was determined at 540nm on a spectrometer. The culture fluid was centrifuged at 5000rpm for 15min (Oguntimhin, 1993). The cell-free culture supernatant obtained served as the source of crude enzyme. The supernatants were assayed amylase.

Effect of Starch Concentration on Growth and Amylase Production by *Lactobacillus plantarum* Strains.

The growth medium consisted of MRS broth in which the D-glucose was replaced with soluble starch at 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v) with the pH adjusted to 6.0. The growth and amylase production for different starch concentrations were determined as previously stated.

Effect of pH on Growth and Amylase Production:

The growth medium consisted of MRS in which glucose was replaced to each of the following PH values (2, 3, 4, 5, 6, 7, 8 and 9) using citrate phosphate buffer.

Effect of Temperature on Growth and Amylase Production by the Isolates:

The same medium as above was used at pH 6.0. Duplicate flasks were incubated at a temperature range of 20, 30, 40, 50, 60, 70, and 80°C for 24 h. Growth and amylase production determination was carried out as previously stated.

Characterization of the Enzymes

Effects of Temperature and pH on Amylase Activity:

One tenth of 1mL of culture supernatant was added to 0.8 mL substrate consisting of 1.2% (w/v) soluble starch in 0.1mL phosphate buffer at pH 6.0. The enzyme-substrate mixture was incubated at 20, 30, 40, 50, 60, 70, and 80°C for 10 min, adding 0.1mL of 5M NaOH to stop reaction. The amount of reducing sugar produced was estimated using the DNSA method as previously stated. For pH, the same procedure was used but at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 using citrate phosphate buffer (Sen and

Charchrabarty, 1986).

Effect of Cations and Inhibitors on Amylase Activity: One tenth of 1ml of culture supernatant was added to 0.8mL of 1.2%(w/v) soluble starch in 0.1M phosphate buffer at pH6.0. To this mixture 0.1, 0.2, 0.3, 0.4 and 0.5M of the cation used and 0.01,0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1Mm of the inhibitors used were separately added. The cations and the inhibitors used are K^+ , Mg^{2+} and Mn^{2+} ; and EDTA, and 2, 4, -DNP, respectively (Arekemase *et al.*, 2020). The activity of the amylase was then assayed as previously described.

RESULTS

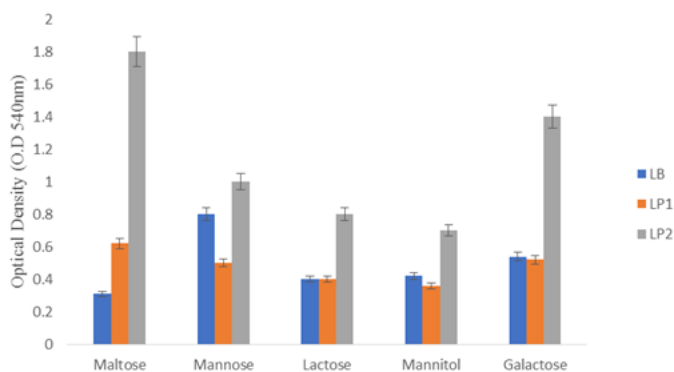


Figure 1 a. Effect of sugars on the growth of the bacterial Isolates. Data represent mean \pm SD ($n = 3$); $P < 0.05$.

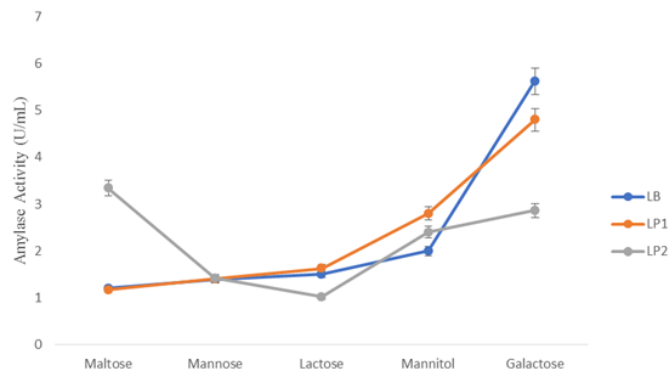


Figure 1b. Effect of carbon sources on the Amylase Production by the bacterial Isolates. Data represent mean \pm SD ($n = 3$); $P < 0.05$.

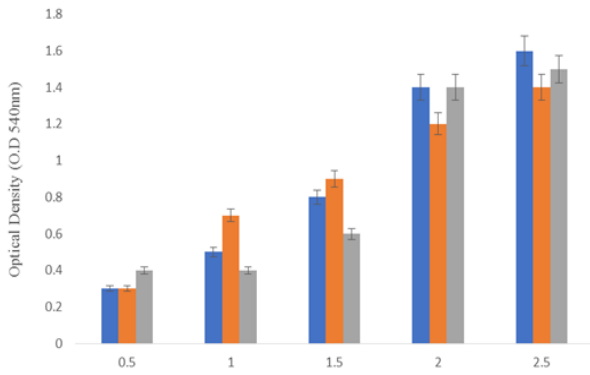


Figure 2a. Effect of different Concentrations of Starch on the growth the Bacterial Isoaltes. Data represent mean \pm SD ($n = 3$); $P < 0.05$.

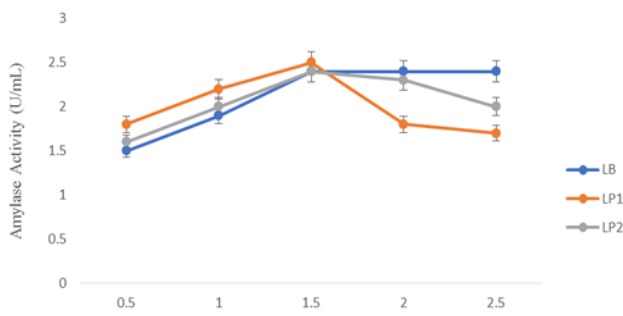


Figure 2b. Effect of different Concentrations of Starch on the Amylase production by the Bacterial Isoaltes. Data represent mean \pm SD ($n = 3$); $P < 0.05$.

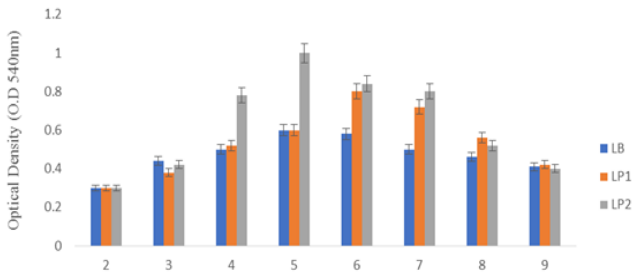


Figure 3a. Effect of different pH on the growth the Bacterial Isoaltes. Data represent mean \pm SD ($n = 3$); $P < 0.05$.

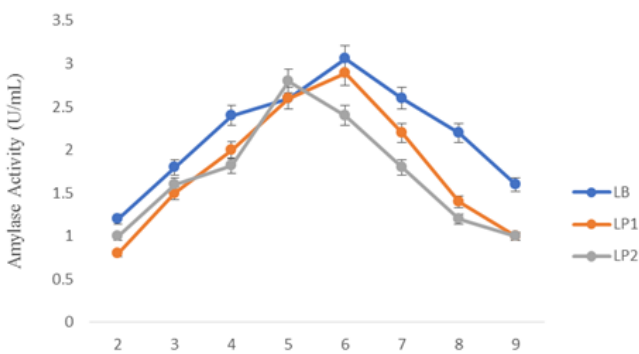


Figure 3b. Effect of different pH on the Amylase production by the Bacterial Isoaltes. Data represent mean \pm SD ($n = 3$); $P < 0.05$.

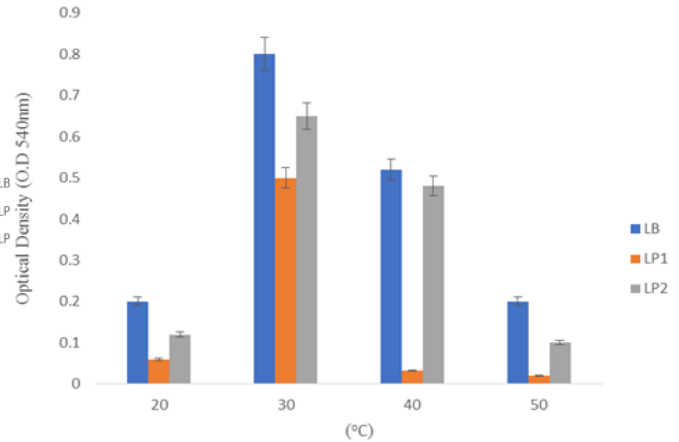


Figure 4a. Effect of different Temperature on the growth the Bacterial Isoaltes. Data represent mean \pm SD ($n = 3$); $P < 0.05$.

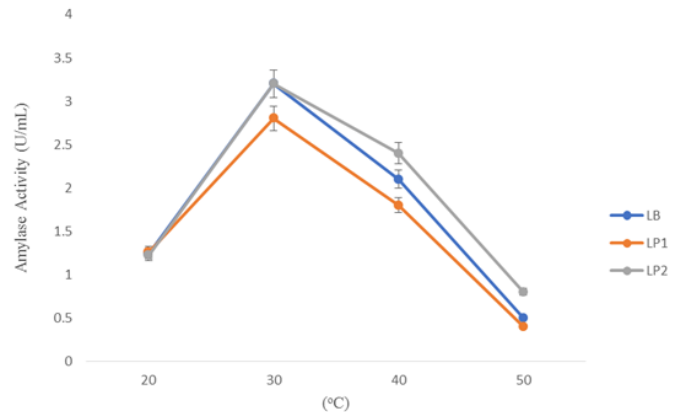


Figure 4b. Effect of different temperature on the Amylase production by the Bacterial Isoaltes. Data represent mean \pm SD ($n = 3$); $P < 0.05$.

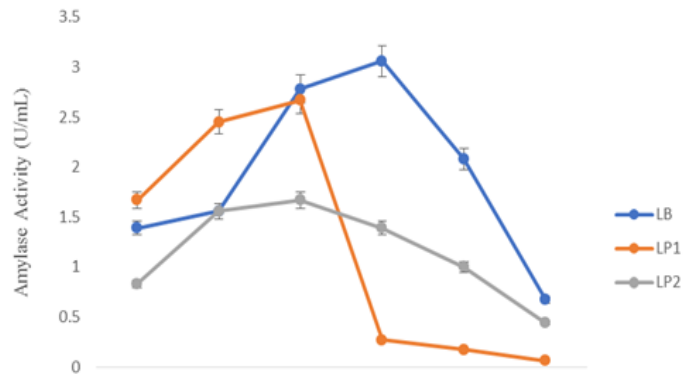


Figure 5. Effect of different temperature on the Amylase Activity. Data represent mean \pm SD ($n = 3$); $P < 0.05$

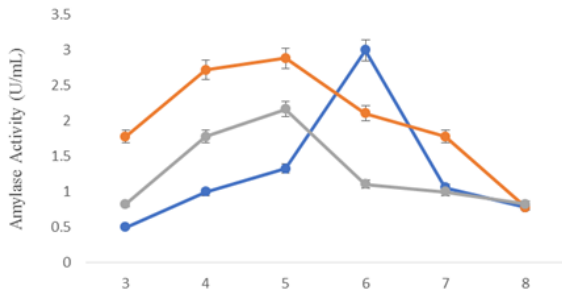


Figure 6. Effect of different pH on the Amylase Activity. Data represent mean \pm SD ($n = 3$); $P < 0.05$

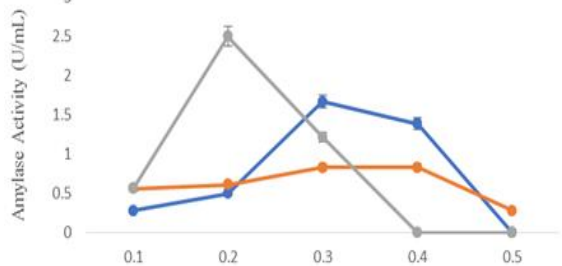


Figure 7a. Effect of different K⁺ on the Amylase Activity (Cation concentration in Molarity). Data represent mean \pm SD ($n = 3$); $P < 0.05$

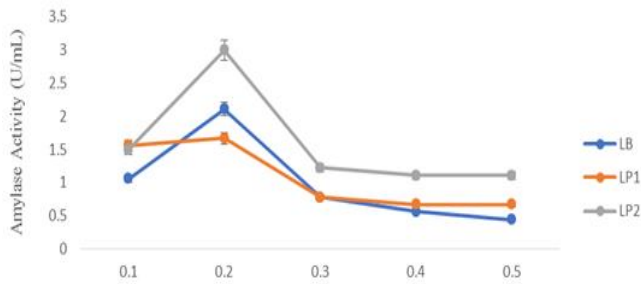


Figure 7b. Effect of different Mg²⁺ on the Amylase Activity (Cation concentration in Molarity). Data represent mean \pm SD ($n = 3$); $P < 0.05$

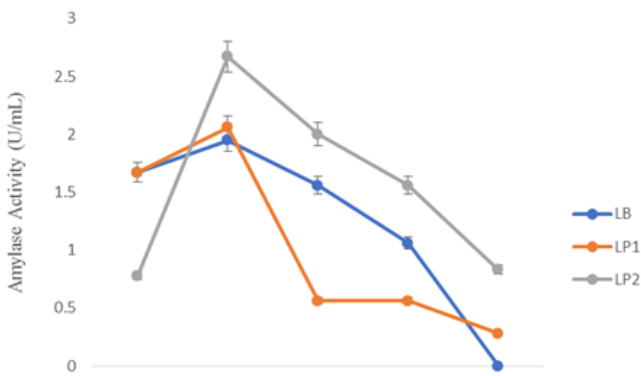


Figure 7c. Effect of different Ca²⁺ on the Amylase Activity (Cation concentration in Molarity). Data represent mean \pm SD ($n = 3$); $P < 0.05$

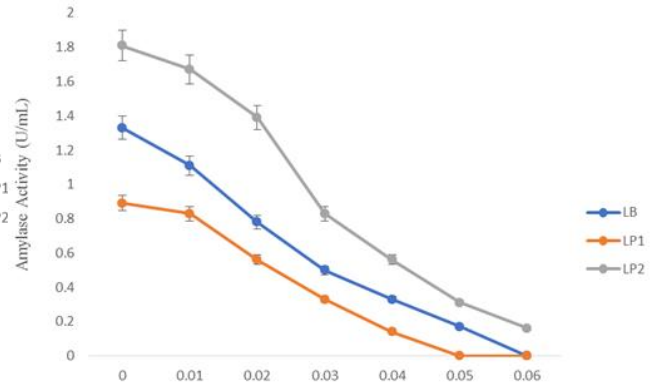


Figure 8a. Effect of different concentrations of EDTA on the Amylase Activity (Inhibitor concentration in milliMolarity). Data represent mean \pm SD ($n = 3$); $P < 0.05$

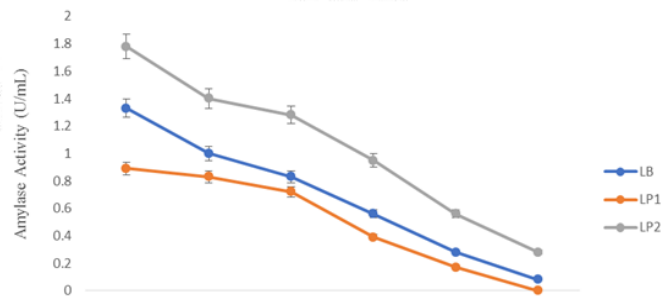


Figure 8b. Effect of different concentrations of DNP on the Amylase Activity (Inhibitor concentration in milliMolarity). Data represent mean \pm SD ($n = 3$); $P < 0.05$

DISCUSSION

The presence of amylase in bacteria, lactic acid bacteria inclusive, has been reported (El-Fallal, 2012; Ekka and Namdeo, 2018). However, because it was believed that *L. plantarum* strains do not produce amylase, integration of alpha-amylase gene of *B. stearothermophilus* into the genome of *L. plantarum* was carried out. A natural amylolytic strain of *L. plantarum* was isolated from fufu and ogi. Investigation of the properties of the enzyme showed that it is extracellular. (Prajesh *et al.*, 2016).

Carbon sources have been showed to have different effects on the growth and amylase production as shown in Figures 1a and b. The different carbon sources did not only support growth but they were also responsible for amylase production. These results agree with the findings of Gunkova *et al.* (2021). The amounts of growth and enzyme production detected are comparable with that of soluble starch (Figures 2 a and b). The findings in relation to the effect of pH and temperature agree with the results of Prajesh *et al.*, (2016) and Aquilar *et al.*, (2000) (Figures 3 and 4 a and b).

In the study of effect of temperature on amylase activity, an increase in activity was observed between 30 °C and 50 °C for LP1 and LP2 and between 30 °C and 60 °C for LB. There was a decrease in the activity of this enzyme between 50 °C and 80 °C for LP2 and between 60 °C and 80 °C for LB. The enzyme behaviour observed here might be linked with the specificity of enzyme to temperature. An enzyme has a specific temperature at which it acts best and below and above which the activity of the enzyme decrease. This trend of activity has been reported for *Bacillus* sp. (Bertrand *et al.*, 2011; Bankefa *et al.*, 2022). Similar observations have been noted for bacteria species (Ilori *et al.*, 1995; Bertrand *et al.*, 2011; Arekemase *et al.*, 2020). The decreased in activity of amylase at temperature beyond the optimal

temperature could be explained based on possible denaturation of the amylase at higher temperature (Sen and Charkrabarty, 1986; Ilori *et al.*, 1995). Enzymes are proteins, the peptidyl bonds of which could easily be broken down at high temperatures.

The effect of pH on amylase activity was studied. Increase in the amylase activity was observed between pH 3.0 and 5.0 for LP1 and LP2 and between pH 3.0 and 6.0 for LB. There was decrease in the activity between pH 5.0 and 8.0 for LP1 and LP2 and at pH 6.0 for LB. The degree of acidity and alkalinity has been reported as factor affecting the activity of amylase (Adesulu-Dahunsi *et al.*, 2022; Neelam *et al.*, 2021). The decrease in the activity of amylase after the attainment of the pH optimum for activity was as a result of the fact that the isoelectric point of the enzyme had been exceeded (Novák and Havlíček, 2016). Similar observations have been recorded by several workers on amylase activity (Sen and Charkrabarty, 1986; Sanni, *et al.*, 1992).

Activity of this amylase in relation to different concentrations of cations was investigated. It was discovered that the activity amylase increased between 0.1M and 0.2M for the enzymes in reaction mixtures containing Mg²⁺ and Mn²⁺. The activity then decreased between 0.2M and 0.5M for the two cations. However, for K⁺, the activity of amylase between 0.1M and 0.3M for LB and LP2 and decreased between 0.3M and 0.5M. These results agreed with that of Arekemase *et al.*, (2020) and Prajesh *et al.*, (2016). They reported that Ca²⁺, Mg²⁺ and K⁺ have stimulating effects at varying concentrations. LP1 had the highest value of Km while LB had the least value Km value. LB therefore has the highest affinity for the substrate.

Manning and Campbell (1961) had earlier reported that calcium ion acted as a stimulant for amylase activity. Others workers have also observed the stimulatory effect of Ca²⁺, Mg²⁺ and K⁺ at varying concentrations. It has also been reported that these can be used as protectors of alpha amylase in animals, plants and microbial origin against denaturation and proteolytic degradation (Manning and Campbell (1961)

Investigation of EDTA and 2, 4-DNP indicates that these compounds have inhibitory effects on amylase. Amylase activity decreased progressively with inhibitor concentration from 0.1mM upward until all activity of the enzyme was lost. This result is in agreement with observations on the effect of EDTA and KNC on amylase activity (Ajayi *et al.*, 2015). This inhibitory effect of these compounds might be as a result of competition for active site on the starch molecule due to the similarity in their molecular arrangement. However, Manning and Campbell (1961) reported that the addition of Ca²⁺ to the reaction mixture can remove the effect of EDTA and thereby allow for full enzyme activity.

The K_{max} and V_{max} of the enzymes were determined. LP1 had the highest value of K_{max} and this is an indication of lowest affinity for the substrate used while LB had the lowest Km value, which also shows that LB has the highest affinity for the substrate (Zusfahair *et al.*, 2020).

Conclusion

Different factors affect the growth and enzyme production by the isolates in this study. It was observed that carbon and nitrogen availabilities impact significant growth and amylase production by the isolates. Our study also established inhibitory abilities of EDTA and 2,4 DNP on the amylase activity. The affinity of the enzyme to the substrates was also low. The organisms in this are potential source of amylase for industrial applications.

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