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Research Article

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PURIFICATION AND CHARACTERIZATION OF AMYLASE PRODUCED FROM PROBIOTIC *LACTOBACILLUS PLANTARUM* CS FOR INDUSTRIAL APPLICATIONS

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ABSTRACT

Previous studies have demonstrated that probiotic Lactobacillus plantarum CS was able to generate an appreciable amount of extracellular amylase, hence the need to purify and characterize it. The aim of the study was to purify and characterize crude amylase from probiotic *Lactobacillus plantarum* CS for its industrial applications Three purification steps including ammonium sulphate precipitation, ion exchange chromatography on carboxymethyl sephadex and gel filtration on Sephadex G-75 were utilized. The homogeneity of the purified enzyme was confirmed using sodium deodocyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The purified amylase was characterized on different parameters including substrates hydrolyses, pH and temperature activity and stability profiles. The general purification elution profile revealed two different peaks of amylase activities with outstanding one having a molecular weight of 59.7kDa. Its purification fold was 4.0 with specific activity of 16.44U/mg protein and enzyme yield of 3%. Temperature optimal activity and stability was at 40^0C and 7.5 for pH activity and stability. Mangenese (Mn^{2+}) (135.17%), tween 80 (128.30%) and some food condiments garlic, thyme, ginger, and tumeric) significantly ($p > 0.05$) enhanced amylase activity (≥262.40%). However, selenium (Se^{4+}) and hydrogen peroxide (H_2O_2) were observed to have greatest inhibiting effect (≥30.9%) on the enzyme. Substrate hydrolysis profiles showed that the amylase hydrolyzed all the test starchy substrates with the highest hydrolytic potential on indigenous sweet potato starch (Km value/ Vmax of 1.33mg/ml/ 7.89ml). The rate of hydrolysis of other test substrates had yam> rice>cassava>corn with km values ≤ 4.0mg/ml and Vmax ≤ 25ml. The obtained results gave an insight that amylase produced from *Lactobacillus plantarum* CS met with the possessed properties suitable for any industrial application especially in food.

Keywords: amylase, characterization, *Lactobacillus plantarum* CS, purification.

INTRODUCTION

The industrial applications of enzymes are increasing nowadays. They are utilized in the various industries such as food, cosmetic, pharmaceutical, poultry and agricultural industries. Enzymes control and speed up reactions in order to quickly and accurately obtain a valuable final product. They also aids in detoxification and breaking down food particles during digestion.

The produced amylase can be used either in a crude or purified form. However, purified amylase is usually preferred as it increases the enzyme specific activity which can be controlled to favor optimal product yield. It is crucial to purify crude amylase in order to reduce the concentration of contaminants such as suspended particles and other undesirable proteins. Purification of amylase is also necessary especially for application where purity is highly needed. Some researchers adopted several purification steps such as ammonium sulfate precipitation, ultra filtration, affinity column chromatography, ion exchange chromatography and gel filtration chromatography (Kosanović *et al*., 2017). Ammonium sulfate precipitation is the most commonly used methods for purification of protein in large and laboratory scale (Chaudhary *et al*., 2006). It separates proteins by altering their solubility in the presence of a high salt concentration. Ion-exchange column chromatography separates molecules based their surface charges. Gel-filtration chromatography separates proteins based on their varying sizes whereby larger molecules are eluted earlier than smaller compounds. Affinity column chromatography purifies proteins according to their specific affinity towards a ligand. Ultra filtration is a method for concentrating and purifying proteins by their molecular weight (Duong-Ly and Gabelli, 2014a&b; Lim *et al*., 2020).

Despite the fact that purification enhances enzyme specific activity, yet there are several factors

that affect its activity. The factors include temperature, pH, metal ion and substrate concentration. These factors can activate or reduce enzyme activity through various mechanisms including activation of enzyme specific sites for increase in activity or blocking the sites for reduction in activity. For instance many substances such as metals and detergents can act as effectors or inhibitors (Dogan *et al*., 2006).

Therefore in this study, the previously produced crude enzyme from *Lactobacillus plantarum* CS George-Okafor *et al*. (2022) was subjected to purification and characterization for possible application in the industry including home-grown poultry industry.

MATERIALS AND METHODS

Isolation of the utilized organism: The microorganism, *Lactobacillus plantarum* CS used in the production of the crude amylase was previously isolated from milled-mixed corn-soybean wastes (George-Okafor *et al*., 2018).

Production of Crude Amylase: The crude amylase was produced using formulated DeMan Rogoa Sharpe (MRS) broth with optimum temperature of 35ºC, pH 6.5 using Mn^{2+}/ Mg^{2+} as a co-factor and natural rain water a solvent for preparation of fermentation medium (George-Okafor *et al*., 2022).

Purification of Crude Amylase: The three purification steps was adopted as stated below;

Ammonium Sulphate Precipitation: The crude amylase (300ml) was first precipitated with 90% saturated ammonium sulphate at 4ºC for 2h. Thereafter, the precipitate amylase was recovered by centrifugation at 10,000 rpm for 20 min at 4ºC using a centrifuge. It was reconstituted with 80ml of 50mM phosphate buffer (pH 6.5) and desalted at 4ºC against the same buffer via dialysis with dialysis tubing of 3.5k MWCO to obtain the final concentrated volume of 40ml which was further purified. Prior to ion-exchange, the dialyzed amylase was assayed for activity as described by Ekka and Namdeo (2018).

Ion-Exchange Chromatography: The resin carboxymethyl sephadex (Sigma Aldrich) was first prepared by dissolving its 5g in 300 ml of sterile distilled water mixed with 0.1% of Na-azide. The gel was allowed to swell in the dark overnight. A column (3.0×30cm) was stuffed with small amount of glass wool within the tap region prior to the addition of small volume of buffer (pH 6.5) in it. With the taps slightly opened, the swollen gel was slowly packed into the already prepared column, avoiding air bubbles. The packed resin was then washed at determined flow rate (0.5ml/min) with 120ml of 10mM HCl (pH 3.5) until the effluent had the same pH as the HCl. Thereafter, it was then equilibrated with 100ml of citrate phosphate buffer (pH 6.5) prior to loading with the recovered dialyzed amylase.

The loaded enzyme (40ml) was initially eluted with 200ml of 0.2M citrate phosphate buffer (pH 6.5) at a flow rate of 0.5ml/min for 10ml/fraction. Further elution with a linear gradient of 0.5M NaCl was carried out at the same flow rate. The observed active fractions were pooled and dialyzed at 4ºC against 5M citrate buffer (pH 6.5) solution for gel filtration.

Gel-Filtration Chromatography: The concentrated enzyme (5 ml) was then loaded on a glass column (1.5 cm × 60 cm) packed with already swollen sephadex G-75 (Sigma, USA) with 0.2M citrate phosphate buffer (pH 6.5). Protein was eluted with 0.2M citrate phosphate buffer (pH 6.5) at a flow rate of 0.5ml/min and the most active fractions were pooled and analyzed as earlier stated.

Protein Determination of Amylase: The protein content of the enzyme was determined using Folin-phenol reagent according to Lowry *et al*. (1951), using bovine serum albumin as a standard.

Homogeneity Test and Estimation of Molecular Weight: The molecular weight of the purified amylase was determined as described by Laemmli (1970), using SDS-polyacrylamide gel electrophoresis. The sample was boiled for 5 min in a dissociation 0.05M phosphate buffer of pH 7 containing 2% SDS and 5% 2-mercaptoethanol. Thereafter, the gel was stained with 0.25% Coomassie brilliant blue R-250 solution followed by destaining and drying (RAPIDRY-MINI). The authentic protein markers (BioLabs, New England) used contained of phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α lactalbumin (14.3 kDa).

Characterization of the Purified Amylase: Purified amylase was characterized based on common factors that influence the enzyme activity and they were described below:

The Effect of Temperature on the Amylase Activity and Stability: This was determined by incubating equal volume (1ml) of enzyme and 1% starch solution in phosphate buffer (pH 6.5) at varying range of temperatures of 10 to 100ºC. After 30min incubation, the amylase activity was assayed using Dinitrosalicylic acid (DNSA) as described by (George-Okafor *et al*., 2022). One unit of amylase activity was defined as the amount of amylase that hydrolyzed starchy compounds to release one microgram of glucose in a minute, under assayed condition. The thermostability profile of the enzyme was determined by pre-incubating 1ml of each enzyme for 10min at different temperature range of 10 to 100ºC prior to reincubation with equal volume of the starch substrate at 40ºC for 30min. Thereafter, the residual activity of the enzyme was determined as earlier stated.

The Effect of pH on the Amylase Activity and Stability: The 1% starch solutions of different pH (3-12) were first prepared with 0.2M of three different buffers (citrate phosphate, phosphate and NaOH-phosphate buffers). Thereafter the enzyme activity was determined by incubating equal volume of each enzyme with that of 1% starch solution of different

pH of 3-12 at optimal temperature of 40ºC for 30min (Singh *et al*., 2012). The pH stability of the enzyme was measured by pre-incubating the amylase with each 0.2 M of citrate phosphate, phosphate and NaOH-phosphate buffers of pH of 3 to 12 prior to reintubation with equal volume of the starch substrate at 40ºC for 30min. Then residual activity was determined as described earlier.

Effect of Metal Salts on Amylase Activity: The following metal salts $MgSO_4$, $MnSO_4$, $AgNO_3$, KNO_3, $CaCl_2$, Na_2CO_3, $HgCl_2$, Se $FeSO_4$, $ZnCl_2$, Pb $(NO_3)_2$, $CuSO_4$ and $CdCl_2$ were assayed for their effects on amylase activity. Each enzyme (1ml) was separately pre-incubated at 40ºC with the individual metal salts (5 mM) in 0.2M phosphate buffer of optimal pH 7.5. The residual enzyme activities were determined. Reaction mixture without any metal ion served as control.

Effect of Modulators on Amylase Activity: Chemical modulators (5mM) assayed were Ethylene diamine tetraacetic acid, sodium dodeocyl sulphate, tween 80 and H_2O_2. The natural modulators were garlic, ginger, turmeric and thyme that were selected based on their utilization as ingredients in some indigenous industrial production that require enzyme application. Garlic, ginger, turmeric were peeled and sliced into pieces. They were dried under mild sun, homogenized using home blender (Corona), sieved with muslin cloth to obtain dry powdered substrates. Thereafter, 10% (w/v) of the prepared natural modulator was solubilized with sterile distill water and agitated under shake-flask for 48h. It was filtered using sterile Whatman No. 1 filter paper and then concentrated to 50ml using a water bath at 30ºC to obtain the concentrated extract utilized for the assay (Erhimu *et al*., 2019). Pre-incubation of the enzyme with each modulator at room temperature for 10min was initiated. Then the reaction mixture contained equal volume of the enzyme with starch solution of pH 7.5 and it was re-incubated for 30min at optimal temperature of 40ºC. Reaction mixture without any modulator served as control.

Relative Rates of Substrates Hydrolysis by Purified Amylase: One percent (1%) of each indigenous starch substrates (yam, sweet potato, corn and cassava) was subjected to hydrolysis by the purified enzyme. The residual enzyme hydrolyzed activity was determined and the optimal concentration (0.5-10%) of the most hydrolyzed substrates was further evaluated, following DNS method as earlier stated. Soluble starch served as the control.

Statistical Analysis: The data obtained from the test and reference samples were carefully analyzed and compared using IBM Statistical Product and Service Solutions (SPSS), version 18.

RESULTS AND DISCUSSIONS

Purification of Crude Amylase

Ammonium Sulphate Precipitation: The treatment with ammonium sulphate purified the crude amylase to a 1.23 purification fold with specific activity of 5.15U/mg (Table1). However, 1.84 purification fold with specific activity of 34.81 U/mg was reported by Karim *et al*. (2018) with *Aspergillus flavus*. Higher purification fold and specific activity was reported by Madhuri *et al*. (2012) to be 3.86 and 1001 U/mg by amylase produced from *Bacillussubtilis.* Lower purification fold and specific activity could be that the salt (ammonium sulphate) used was not properly stored and this could affect its solubility hence the enzyme will not be completely precipitated from the solution. This is because the salt is at risk of deteriorating in warm and humid environments (Purwanto,2016).

Ion Exchange Chromatography: Carboxymethyl Sephadex (Cm Sephadex) was able to purify the enzyme to 3.39 folds with specific activity of 14.24U/mg (Table. 1). The obtained purification fold (3.39) can be compared to that obtained by Irshad *et al*. (2012) that recorded purification fold of 1.2 with the same purification process of amylase from *Ganoderma tsuage*. The increase in purification fold is an indication that the resin used had a better specificity hence higher purification was achieved.

Gel-Filtration Chromatography: Two peak regions at 2-7 and 11- 13 fractions were observed during the chromatography (figure 2). The two peak regions suggest two different amylase enzymes, possible an isoenzymes. The result is similar with the findings of Mohamed (2004) that revealed 3 isoenzymes after ion exchange chromatography. The amylase had purification fold of 4.0 which can be compared with the purification fold of 3.80 of thermostable alpha amylase from *Bacillus subtilis* (Aladejana *et al*., 2020). However lower purification fold of 2.67 of amylase from *Geobacillus stearothermophilus* was reported by (Snehi *et al*.,2021). There was increase in the specific activity after each purification step indicating the adequacy of the employed purification methods.

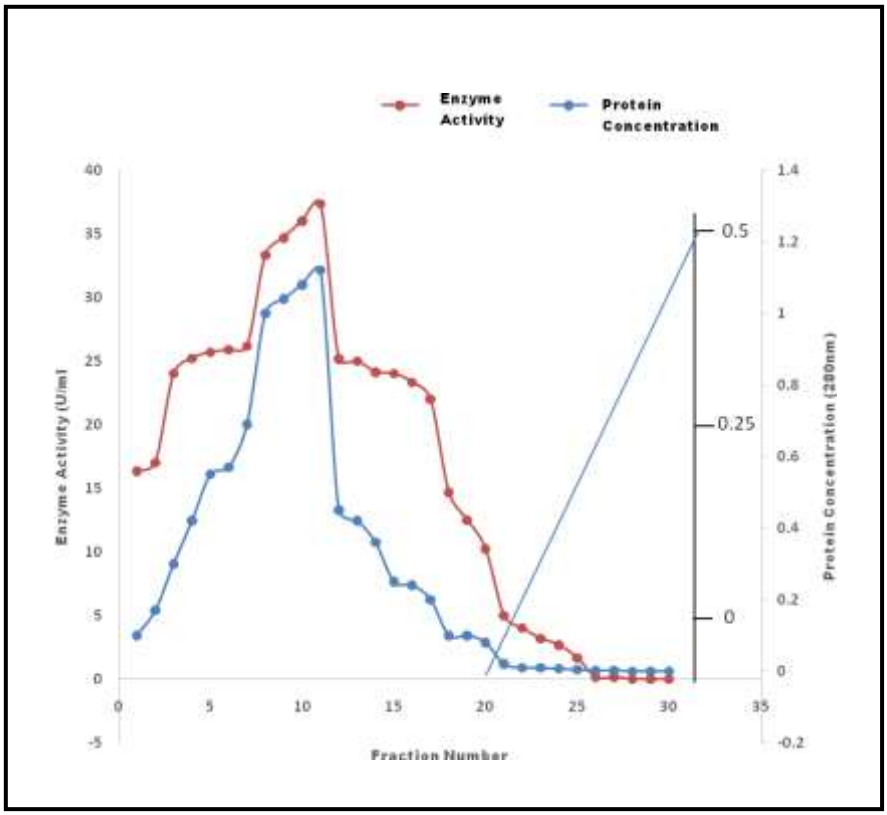

Figure 1. Enzyme Elution Profile of Ion Exchange Chromatography on Carboxymethyl Sephadex.

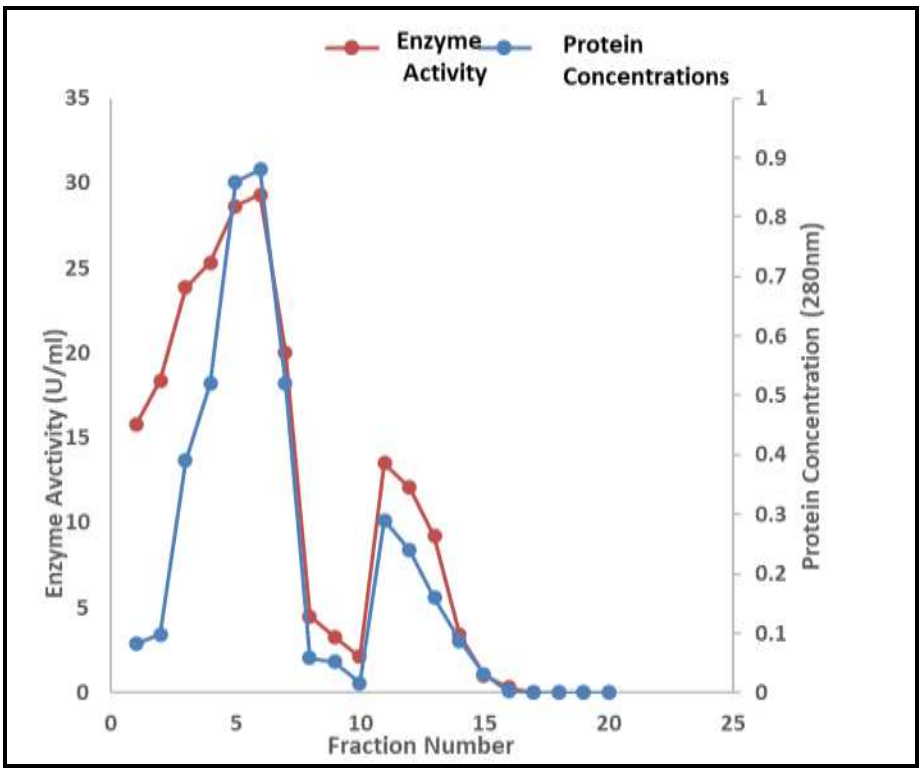

Figure 2. Elution Profile of Amylase on Sephadex G75 (Gel Filtration).

Homogeneity Test and Estimation of Molecular Weight: The homogeneity test result shows numerous protein molecules (bands) with the crude enzyme sample. These bands were reduced to only 6 with Carboxymethyl Sephadex purification. Final purification with Sephadex G-75 indicated a single band with molecular weight of 59.7 kDa (Figure 3). A single band was also recorded by Rathour *et al.* (2020) with lower molecular weight of 44kDa using purified amylase from *Shewanella* sp. However,

single band with higher molecular weight of 73kDa from purified amylase of *Bacillus mojavensis* was also reported (Ozdemir *et al*.,2018).

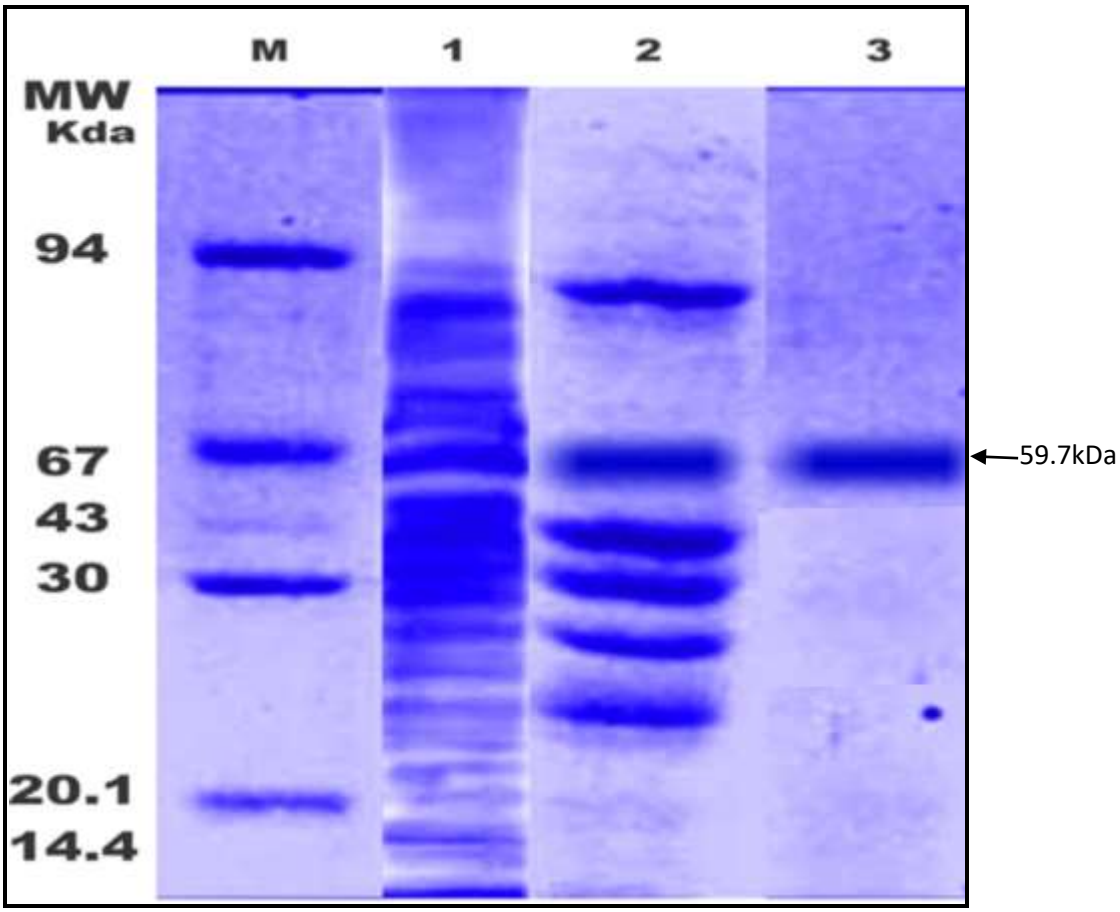

Figure 3. SDS-PAGE of the Purified Amylase with the Marker Protein

Legend: M: Marker proteins – Phosphorylase B (94kDa), Bovin serum albumin (67kDa), Chicken egg ovalbumin (43kDa), Carbonic anhydrase (30kDa), Trypsin inhibitor (20.1kDa) and lactalbumin (14.4kDa). 1: crude enzyme, 2: Carboxyl methyl sephadex purified enzyme, 3: Sephadex G75 purified enzyme

Table 1. Summary of the Purification Steps of Amylase from *Lactobacillus plantarum* CS

Purification Step	Protein Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield of Activity (%)	Purification fold
Crude extract	300	9.17	11550	4.19	100	1.00
Ammonium sulphate (90% saturation)	80	6.09	2510.40	5.15	21.74	1.23
Carboxyl-methyl sephadex	20	1.90	541.40	14.24	4.69	3.39
Sephadex G-75	5	1.63	119.00	16.44	1.03	4.00

Effect of Temperature on the Amylase Activity and Stability: The optimum temperature activity and stability was observed at 40^{o}C. The activity decreased as the temperature was increased (Figure 5). The optimum temperature for amylase activity at 40^{0}C obtained from this study is similar with that of Tatah and Otitoju (2015) but contrary with Cordeiro *et al*. (2002) report that recorded optimum temperature activity and stability at 70^{0}C.

The decrease in the activity at higher temperature indicated that the enzyme can be affected by heat. This could be attributed to the findings of Lu *et al*. (2010) which showed that the rate of catalyzed reaction increases at optimum temperature with decrease at higher temperature due to the denaturation of the enzyme which is protein in nature.

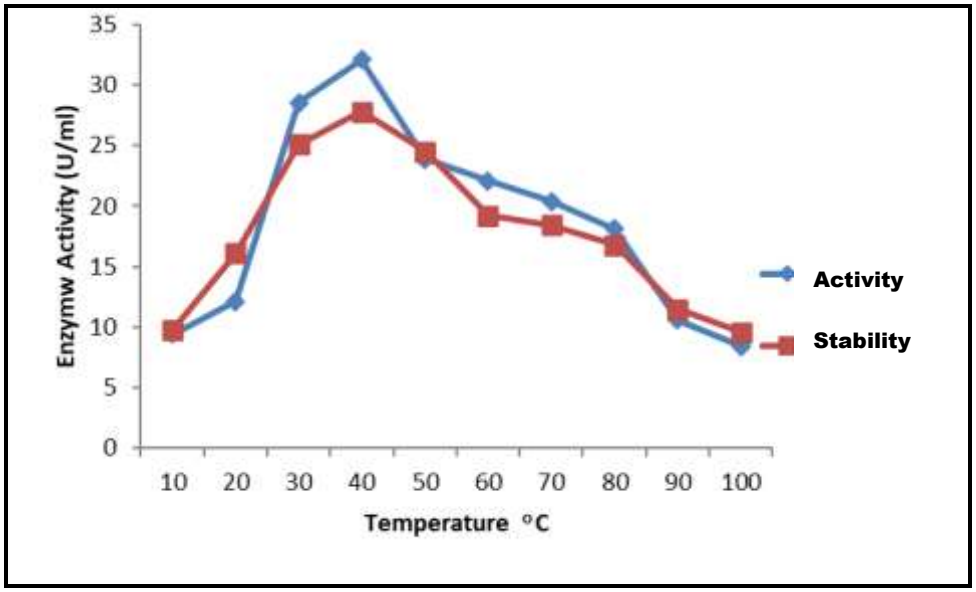

Figure 4. Effect of Temperature on the Amylase Activity and Stability

Effect of pH on the Amylase Activity and Stability: The enzyme had activity at pH of 7.5(Figure 4). The activity of the amylase drastically reduced after the pH of 7.5; an indication that amylase from *L. plantarum* CS is pH dependent and can be classified as a neutrophilic enzyme having their optimal activity within 6.5-7.5 pH range. The result is similar with the finding of Cordeiro *et al*. (2004) which indicated *Bacillus* sp. amylase activity at pH 7.5. However, the result was contrary with the results of Hye-Yeon *et al*. (2016) who report a pH optimum of 3.5 *Lactobacillus plantarum* amylase.

The optimum pH stability of *Lactobacillus plantarum* CS amylase was the same as recorded for activity (Figure 4). Bano *et al*. (2011) reported pH stability of amylase from *Bacillus subtilis* at 7.5. In contrary, lower pH stability of 5.0 was recorded (Kanpiengjai *et al*., 2015b).

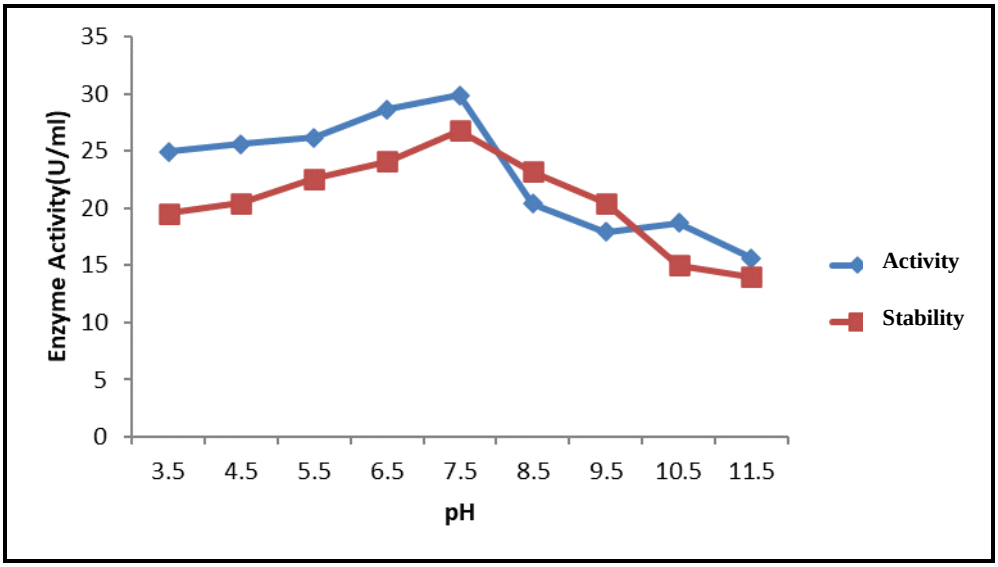

Figure 5. Effect of pH on the Amylase Activity and Stability

Effect of Metals Salts on Amylase Activity: Among the metals assayed, only Mn^{2+}, Mg^{2+} and Fe^{2+} enhanced the activity of the amylase (Figure 6). Mn^{2+} was able to enhance amylase activity most (32.44U/ml). The enhancement by these metals revealed that this enzyme can thrive well and metabolize in the presence of the metal ions. The result is similar with the findings of Ozdemir *et al*. (2018) that indicated higher amylase activity with Mg^{2+} by 105%. In contrary, Mn^{2+} inhibited the amylase activity of *Bacillus firmus* (Zohra *et al*., 2016). The inhibition of Hg^{2+}, Cd^{2+}, Cu^{2+} on amylase were also reported by Ozdemir *et al*. (2018) and Prakash *et al*. (2011). The stability of the enzyme to some of the metal ions suggests its promising potential in detergent industries as the activity would not be reduced in the presence of metals in the detergent.

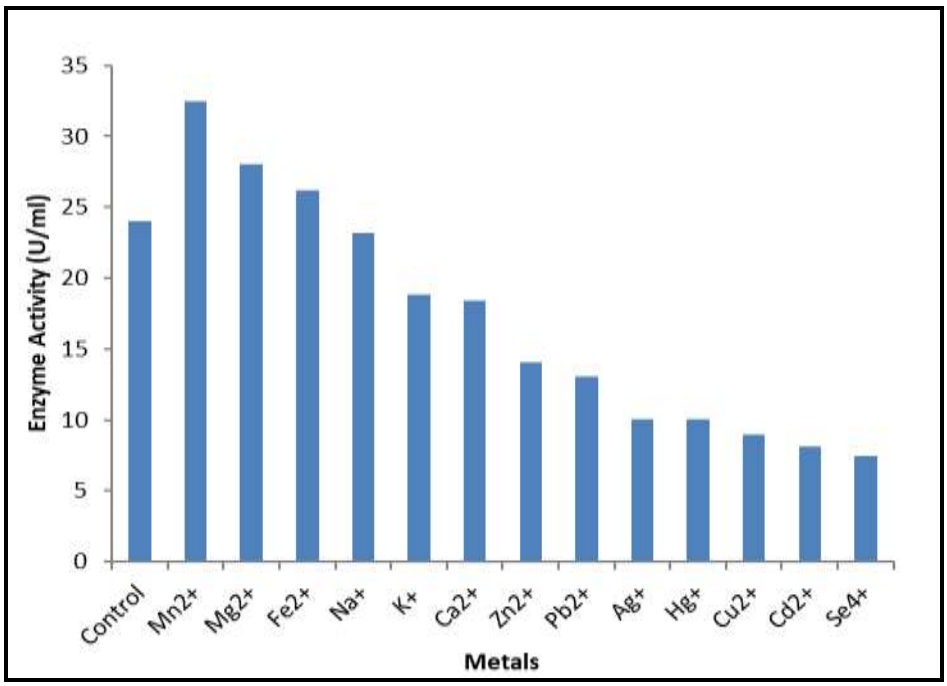

Figure 6. Effect of Metals Salts on Amylase Activity

Effect of Modulators on the Amylase Activity: The result revealed that all the natural modulators assayed significantly enhanced amylase activity at P >0.05(Table 2). All the chemicals except tween 80 inhibited the amylase activity (38.05% - 86.7%). The inhibition was more with H_2O_2 (±38.05%). The enhancement of amylase activity by natural modulators (food spices) is interesting as it shows that the presence of spices on starch foods would not hinder their hydrolytic activity of the functional enzyme. The inhibition of amylase activity by the chemical modulators is similar with the findings of Keskin and Ertunga (2017) and Zohra *et al*. (2016) which showed that EDTA, SDS and H_2O_2 inhibited amylase activity. The inhibition of EDTA is contrary to the result of Sajedi *et al* (2005) which had EDTA as amylase activity enhancer of *Bacillus* sp. KR-8104 enzyme.

Table 2. Effect of Modulators on the Amylase Activity

Nature of Modulators	**Modulators**	**Relative Activity (%)**
Control (Without Modulators)		100.00±0.04
Natural	Garlic	262.40±0.02
	Thyme	265.50±0.08
	Ginger	180.70±0.02
	Tumeric	149.90±0.06
Chemical	Tween 80	128.30±0.16
	EDTA	86.70±0.18
	SDS	52.80±0.06
	H_2O_2	38.05±0.14

$$\text{Relative Activity (\%)} = \frac{\text{Enzyme Activity of Test Modulator}}{\text{Enzyme Activity of Control}} \times \frac{100}{1}$$

A = Ethylenediaminetetraacetic acid, H_2O_2 = Hydrogen peroxide, SDS = Sodium Dodecyl Sulphate

Substrate Hydrolysis Profile of Amylase from *L. plantarum* CS: The amylase was able to hydrolyze the food starch components at 1% concentration (Figure 7). The results of amylase kinetics studies on substrate concentration are stipulated in Figure 8. The obtained Km values from the three hydrolyzed food starch (1.33-4.0mg/ml) are similar to that obtained by Sakač, and Sak-Bosnar (2012), Bano *et al*. (2011) and Narayana and Vijayalakshmi (2008) who recorded Km values of 0.5771mg/ml, 2.68mg/ml and 4.0mg/ml from amylase produced by *Bacillus subtilis* and *Streptomyces albidoflavus*, respectively. However, higher Km value of 15.85mg/ml was reported by Tallapragada *et al*. (2017) for amylase from *Monascuss anguineus*. The Vmax (6.67-25.0U/ml) was lesser than that of Bano *et al*. (2011) which gave Vmax value of 1773 U/ml. However, Sakač and Sak-Bosnar (2012) reported lower Vmax value of 3.31U/ml. This result which had the lowest Km value for potato starch could be the reason for its most hydrolytic efficiency. This can be supported by the report of McDonald and Tipton (2022), which stated

that low Km value results to high efficiency of enzyme and high Km values result to less efficiency.

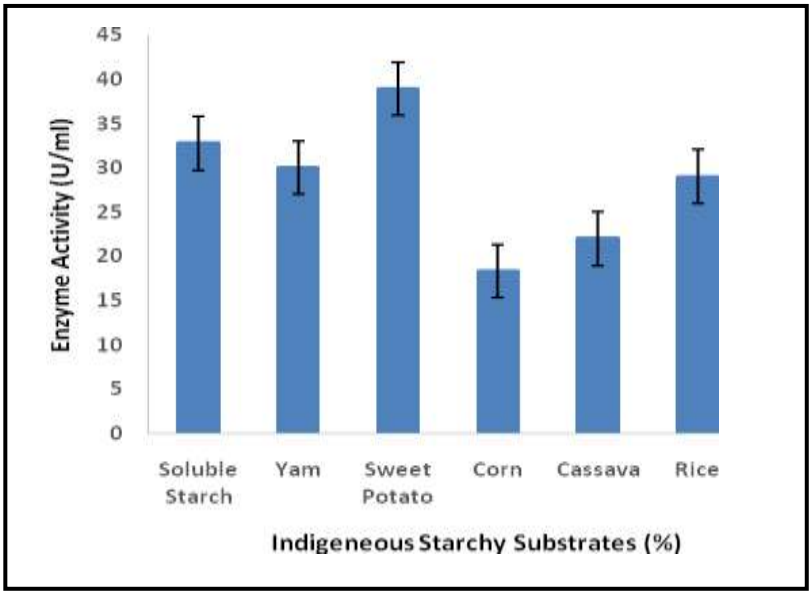

Figure 7: Substrate Hydrolysis Profile of Amylase from *L. plantarum* CS

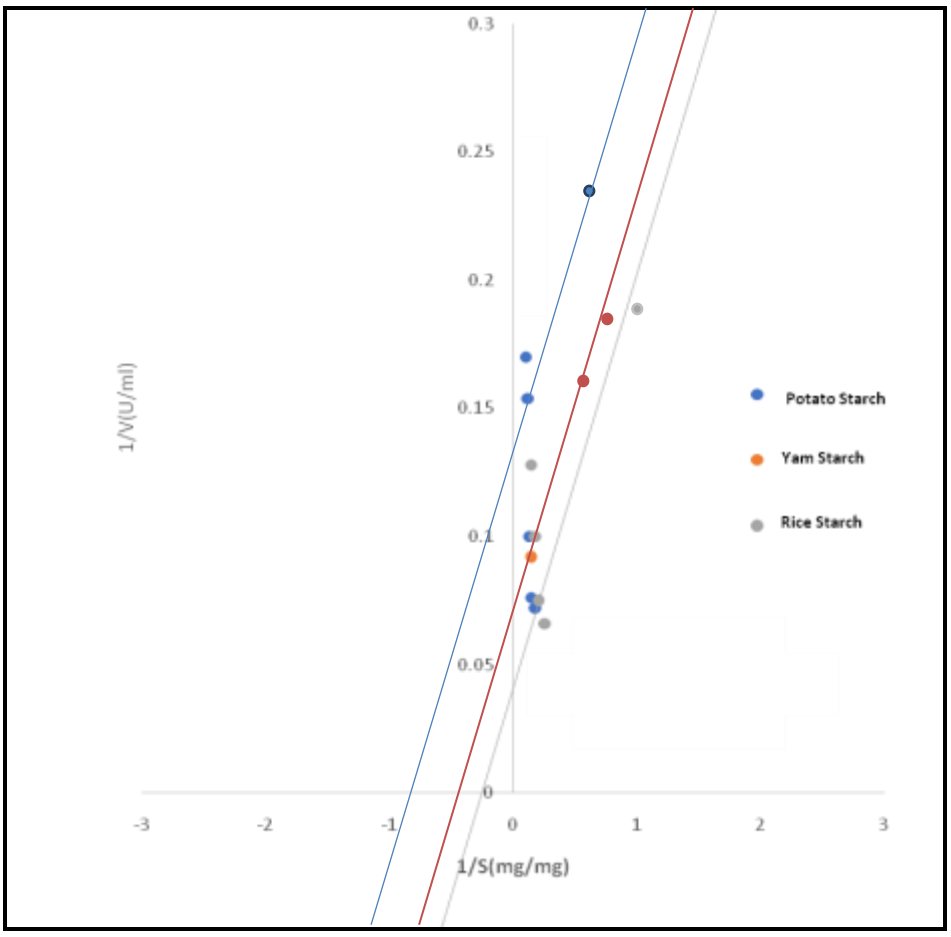

Figure 8: Lineweaver-Burk Plot for the Determination of Km of Purified Amylase for various Carbon- Substrates

CONCLUSION

The crude amylase from *L. plantarum* CS had 4fold purification with gel chromatography and demonstrated stability at 40^{o}C with strong hydrolytic potentials on indigenous substrates, an indication of

its suitability for industrial application especially in food.

DISCLOSURE OF CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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DECLARATION OF INTEREST STATEMENT

To the best of our knowledge, none of the authors have any conflict of interest, financial or otherwise

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