Ceylon Journal of Science 49(1) 2020: 61-69
DOI: http://doi.org/10.4038/cjs.v49i1.7706

RESEARCH ARTICLE

Kinetic characteristics of partially purified invertase from *Citrullus lanatus* Rind

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Received: 16/01/2019; Accepted: 25/01/2020

Abstract: Invertases are enzymes that hydrolyze sucrose to produce equimolar mixture of glucose and fructose. They are widely used in various industrial food applications. The aim of this study was to isolate, partially purify, and characterize invertase from *Citrullus lanatus* rind. Invertase isolated from *C. lanatus* rind was purified to 46.94 folds with 23.19% yield by means of ammonium sulphate precipitation, dialysis and Sephadex G-25 gel filtration chromatography.

The enzyme has an optimum temperature of 50 °C and maximum activity at pH 7 and a relatively high activity at pH 4. Invertase enzyme from *C. lanatus* rind maintained its activity at 50 °C and 95 °C after 20 minutes of incubation. Maximum activity of the enzyme occurred at 0.25 M sucrose concentration. Kinetic parameters, Vmax and Km were 15 mM and 40 µM/min, respectively. *C. lanatus* rind invertase was competitively inhibited by Fe^{2+}, Cu^{2+}, Mg^{2+} and Ag^{2+}, while Co^{2+} enhanced its activity. Zn^{2+} has relatively little or no effect on the activity. Thus, *C. lanatus* rind may be employed as a local source for the production of invertase enzyme.

Keywords: Invertase, *Citrullus lanatus* Rind, Purification, Kinetic Parameters.

INTRODUCTION

Invertase (E.C 3.2.1.26) also known as β-fructofuranosidase is an enzyme which catalyzes the breakdown of sucrose which is a non-reducing disaccharide to fructose and glucose which are reducing monosaccharides (Ahmed, 2008). The mixture of glucose and fructose produced is called inverted sugar syrup (Mobini-Dehkordi *et al*., 2008). Invertases exist in different isoforms in nature and these isoforms are differentiated by their locations, in yeasts cell for example, it is present in two forms as either extracellular or intracellular invertase, while in plants, as three isoforms, each different in their biochemical properties and subcellular locations (Acosta *et al.,* 2000).

Invert sugar consists of an equimolar mixture of fructose and glucose which has been reported to be sweeter and to have lower crystallinity than sucrose (Goosen *et al.,* 2007). Invertases are known to be used in various industrial food applications especially in the preparation of jams and candies, these enzymes are also essential in the production of non- crystallizing creams, artificial honey, lactic acid, ethanol, confectionary (food), in digestive aid tablets, powder milk for infants and other infant foods (Acosta *et al.,* 2000; Phadtare *et al.,* 2004; Sikander 2007). Despite the wide range of application of invertase in various industries, the commercially available invertase is rather expensive, thus limiting the applicability of the enzyme.

Micro-organism are mainly employed in the production of invertase in a process that needs very strict regulation of production conditions and requires high level of purification for taste and health reasons, thereby making the enzyme expensive (Laluce, 1991). Plant enzymes have been reported to be have higher thermal stability than microbial enzymes (Tananchai and Chisti, 2010). Thermostability is a very important prerequisite for industrial applicability of enzymes.

Citrullus lanatus (water melon) is a flowering plant which is vine-like, and from the family *Cucurbitaceae*. It comprises of about 6% sugar, 91% water and the residual portion consisting of vitamins and minerals. Water melon has been reported in several studies to have therapeutic properties, including antihypertensive, anti-diabetic, antioxidant, anti-inflammatory and antimicrobial effects (Arise *et al.,* 2016a; Arise *et al.,* 2016b; Erhirhie and Ekene, 2013). The rind of watermelon rind is usually discarded as waste, although it is edible (Al-Sayed and Ahmed, 2013). The increasing concern about pollution that occurs from agricultural and industrial wastes has stimulated interest in the conversion of waste materials into commercially valuable products such as enzymes, oil, pectin, wine/vinegar e.t.c (Rashad and Nooman, 2009; Sangeetha *et al.,* 2005). Therefore, the aim of the study was to isolate, partially purify and characterize invertase from watermelon rind as well as to determine the kinetic factors and conditions that will maximize the activity of the enzyme which will help boost its suitability for industrial prospects.

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MATERIALS AND METHODS

Plant material

Citrullus lanatus (water melon) was purchased from vendors in Ilorin metropolis, Kwara state, Nigeria. It was authenticated at the Herbarium Unit of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria.

Reagents and Assay Kits

Sephadex G-25 and ethylenediamine tetraacetic acid (EDTA), were products of Sigma Chemical (St. Louis, USA), while sucrose and 3,5-dinitrosalicylic reagent were products of Kem light laboratories, Mumbai, India. All other reagents used were of analytical grade.

Preparation of Plant Extract

The rind of water melon was removed from the whole water melon ball, and the white portion of the rind was carefully scraped into a clean container, cut into small pieces and weighed. Three thousand milliliter (3000 ml) of ice cold sodium phosphate buffer (pH 7) containing 1 mM EDTA, and 50 mM sodium metabisulfite, were added to 1700 g of the rind. A sterilized stainless Maxwell blender placed in a freezer for 24 hours was used to pulverize the mixture of rind and buffer. The entire slurry was filtered using a clean three-layer muslin cloth at 4 °C and then centrifuged at 15000 g for 30 minutes at 1 ºC using a cold centrifuge. The supernatant obtained afterwards was then stored at 4 ºC and it served as the crude enzyme source.

Invertase and protein assays

Assay for invertase activity was carried out using a 5-minute standard test as described by Timerman (2012). The original enzyme source (OES) was appropriately diluted five (5) times by adding 8ml of 50 mM sodium phosphate buffer to 2ml of OES to obtain 10ml of diluted enzyme source (DES). Aliquot volume (0.5 ml) of DES was carefully transferred into a test tube containing 2 ml of substrate solution (composed of 50 mM sucrose in 50 mM sodium phosphate buffer at pH 7) and the reaction was allowed to proceed for 5 minutes, after which the reaction was stopped by denaturation, using the rapid addition of 2.0 ml of alkaline solution composed of NaOH, 3, 5-dinitrosalicylate, and sodium potassium tartrate. The assay tube was transferred to a boiling water bath for 7-8 minutes. The solution was then allowed to cool and diluted to a final volume of 6.1 ml with the addition of 1.6 ml of 50 mM sodium phosphate buffer at pH 7. Absorbance of the assay was measured spectrophotometrically at 540 nm.

Ammonium sulphate precipitation

Ammonium sulphate precipitation was carried out in ice bath using freshly prepared ammonium sulphate crystals. Powdered ammonium sulphate was weighed and slowly added to the crude extract and stirred gently and the solution kept overnight at 4 ^{o}C. After saturation, each precipitate was collected using a centrifuge at 16500 $\times g$ for 30 minutes at 4 ^{o}C. The precipitate was dissolved again using sodium phosphate buffer (pH 7) and then assayed for invertase activity (5 ml buffer for every 5 ml precipitate). The saturation with the highest activity of invertase was further purified. The protein concentration was determined using Biuret method, as described by Gornall *et al.* (1949) with bovine serum albumin (BSA) serving as standard.

Dialysis of precipitated fraction

Ammonium sulphate precipitation fraction was dialysed overnight with intermittent stirring. The dialysis buffer was changed 3 times at 2 h interval. The activity of invertase and protein concentration this fraction were determined as described above.

Gel filtration with Sephadex G-25

The sephadex G-25 gel was pre-equilibrated with sodium phosphate buffer at pH 7, a slurry of the gel was poured into a column filled to 1/4th of its volume with buffer and was allowed to set for 24 hours. The dialyzed fraction was then loaded at the top of the gel and washed down with sodium phosphate buffer, pH 7. The fractions were collected by volume at the interval of 2 ml each with a flow-rate of 37 ml/h. The activity of invertase and protein concentration of each fraction were determined and the active fractions were pooled.

Characterization of *C. lanatus* rind invertase

Determination of optimum pH

Different 50 mM buffers (sodium phosphate, sodium acetate and Tris-HCl buffers) at pH ranging from pH 2-9 were prepared, they were used differently to incubate the enzyme for 30 mins after which the activity of invertase was determined (Bhatti *et al.,* 2006; Amin *et al.,* 2008).

Determination of optimum temperature

Invertase activity was studied for optimum temperature using a method described by Amin *et al.* (2008). Five milliliter (5 ml) of the diluted enzyme source was incubated at temperatures ranging from 10 ºC to 90 ºC for 30 minutes and then assayed for invertase activity.

Effect of heat treatment

The effect of heat treatment on the activity of the enzyme was carried out using a method described by Violet and Meunier (1989). Aliquot volume of enzyme source was heated at 50 ºC and 95 ºC, after which samples were removed at interval of 5 minutes and then assayed for invertase activity. Three milliliter (3 ml) of enzyme source was pipetted into 6 sample bottles. Three milliliter (3 ml) of blank preparation was also pipetted simultaneously into another set of 6 sample bottles; all were placed in a hot water bath at the desired temperature. At 5 min interval, a sample bottle containing enzyme and another containing blank were withdrawn and cooled to room temperature in order to attain equilibrium and then assayed for invertase activity. Residual activity was expressed as a percentage of the activity under the standard assay condition.

Substrate kinetics

Substrate kinetics was done according to the method adapted by Sivakumar *et al.* (2012). Varying concentrations of substrate ranging from 50 mM to 300 mM were prepared. The standard 5 min assay was carried out with each substrate concentration and the absorbance read at 540 nm. Double reciprocal graphs were then plotted to determine the Vmax and Km values of the enzyme.

Effect of some metal ions on invertase activity

The method adapted by Bhatti *et al.* (2006) was used to measure the effect of some metal ions on the invertase activity. Fifty millimole (50 mM) concentrations of each ion were prepared and the reaction mixtures were incubated for 30 minutes, after which the activity of invertase was determined, and compared with the activity of the enzyme in the absence of metal ion.

Statistical Analysis

All data were expressed as the mean of three replicates ± standard error of mean (S.E.M). Statistical evaluation of data was performed by SPSS version 16 using one way analysis of variance (ANOVA).

RESULTS

The three step purification of invertase from water melon rind, gave the following result:

Ammonium sulphate precipitation

As shown in Table 1, the 20-60% saturation fraction had the highest specific enzyme activity (0.633 µM/min/mg), purity (0.99) and percentage yield (68.25%), this is followed by the 20-50% saturation fraction which had 0.513 µM/min/mg specific enzyme activity, 0.80 purity and 48.75% yield. The 20-70% saturation fraction had the lowest level of specific activity, purity and percentage yield (0.232 µM/min/mg, 0.36 and 17.06 % respectively).

Dialysis of precipitated fraction

The dialysate was assayed for invertase activity and the total protein determined. There was an increase in the specific activity of the enzyme after dialysis as undialyzed fraction had 14.46 µM/min/mg while dialyzed fraction had 116.29 µM/min.

Gel filtration

The gel filtration purification profile is presented in Figure 1. Fraction 8 had the highest invertase activity, followed by fractions 9 and 10 respectively. These fractions were pooled together and further characterized. Going down the whole purification profile from crude extract to gel filtered enzyme source, there was an increase in the specific activity of the enzyme while the protein concentration decreased as unwanted proteins were removed through the purification process as shown in Table 2. The gel filtration fraction had the highest value of specific enzyme activity (501.88 µM/min/mg), which eventually gave a final purification fold of 48.94.

Effect of substrate concentration on *C. lanatus* rind invertase activity

Substrate kinetics for the rate of sucrose hydrolysis by *C. lanatus* rind invertase was estimated using a Michealis-Mentens curve (Figure 2). The result showed the activity of the enzyme increased progressively up until 250 mM substrate concentration, after which further increase in substrate concentration did not lead to an appreciable increase in activity of the enzyme. The Lineweaver-Burk plot for hydrolysis of sucrose catalyzed by *C. lanatus* rind invertase (Figure 3) gave 15 mM for the Km value and 40 µM/min for Vmax.

Table 1: Summary of ammonium sulphate precipitation of *C. lanatus* rind invertase

% Saturation	Total protein (mg)	Activity (µM/min)	Specific activity (µM/mg)	Purification fold	% Yield
Crude	67.241	42.916	0.638	1	100
0 - 20%	16.092	19.201	1.193	1.870	44.741
20 - 50%	40.805	20.934	0.513	0.804	48.779
20 - 60%	46.264	29.291	0.633	0.992	68.252
20 - 70%	31.609	7.323	0.232	0.363	17.063

Table 2: Summary of purification process of *C. lanatus* rind invertase from crude extract to gel filtration step.

Fraction	Total protein (mg)	Activity (µM/min)	Total activity (µM/min)	Specific activity (µM/min/mg)	Purification fold	% Yield
Crude	145.402	31.094	1554.682	10.692	1.000	100.00
NH_2SO_4 ppt	51.006	14.757	737.826	14.465	1.353	47.46
Dialyzed	3.305	7.686	384.303	116.293	10.876	24.72
Gel filtration	0.718	7.211	360.546	501.880	46.938	23.19

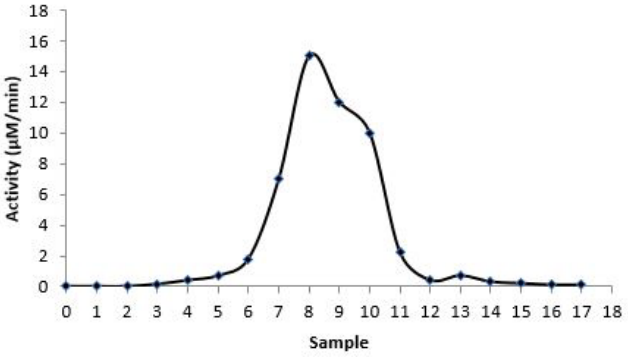

Figure 1: Purification profile of *C. lanatus* rind invertase by sephadex G-25 gel filtration.

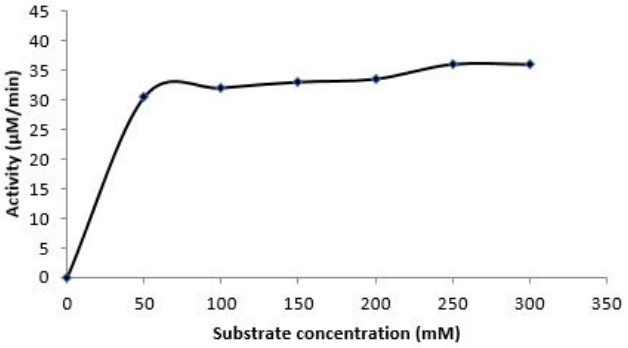

Figure 2: Concentration dependent effect of sucrose (substrate) on *C. lanatus* rind invertase activity.

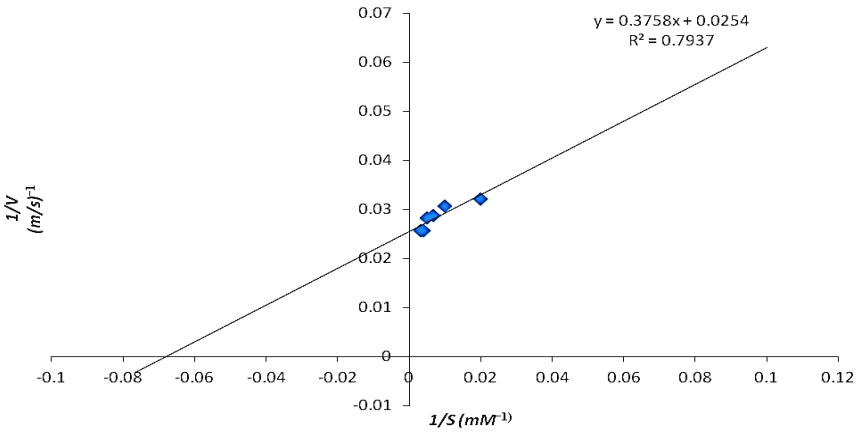

Figure 3: Double reciprocal plot of *C. lanatus* rind invertase activity using sucrose as substrate.

Effect of pH on the activity of invertase from *C. lanatus* rind

The effect of different pH (2-9) on the activity of *C. lanatus* rind invertase was determined. The result showed that the activity of *C. lanatus* rind invertase was at its peak at pH 7, but it also had a very high activity at pH 4 as graphically represented in Figure 4.

Determination of optimum temperature

The graphical representation of the effect of temperature on the activity of *C. lanatus* rind invertase is shown in Figure 5. The peak activity of the enzyme was observed at 50 ºC, with gradual decline in activity as the temperature was increased further.

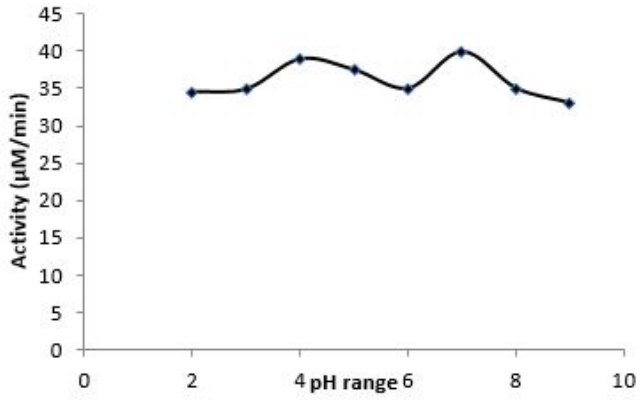

Figure 4: Effect of pH on *C. lanatus* rind invertase activity.

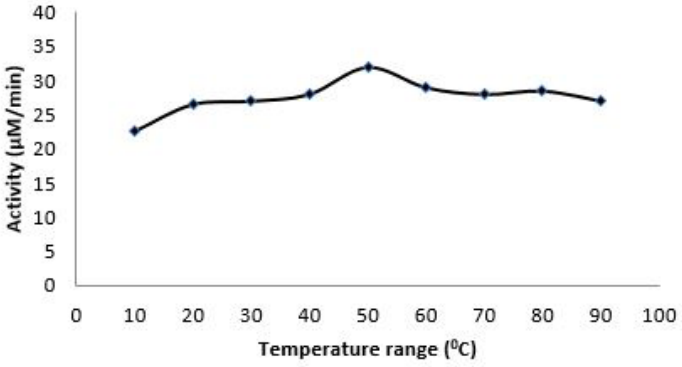

Figure 5: Effect of temperature on *C. lanatus* rind invertase activity.

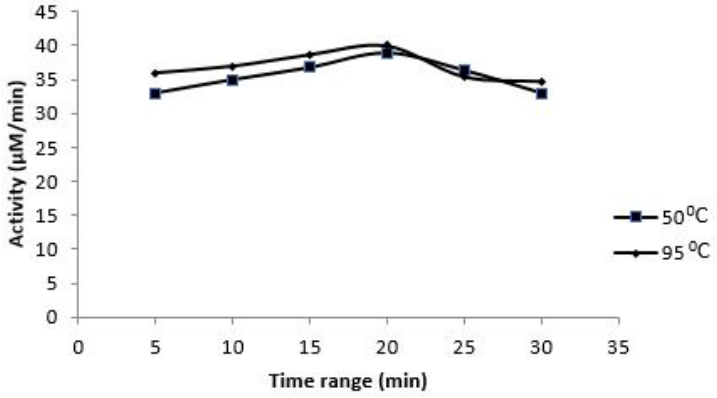

Figure 6: Effect of heat treatment on *C. lanatus* rind invertase activity.

Effect of heat treatment on *C. lanatus* rind invertase activity

The effect of heat treatment at 50 ºC and 95 ºC on the activity of *C. lanatus* rind invertase is represented in Figure 6. The enzyme was extremely thermostable as it retained very high activity after incubation for 20 min at 50ºC and 95 ºC respectively and the activity slightly decreased with further incubation. The optimum activities observed after 20 min incubation at 50 ºC was 30% higher than the activity observed at less than 10 ºC (temperature at which all reactions were carried out) while the activity was 32.5% higher after incubation for 20 min at 95 ºC.

Effect of some metal ions on *C. lanatus* rind invertase activity

The effect of different concentrations of Fe^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} on the rate of hydrolysis of sucrose catalyzed by *C. lanatus* rind invertase was observed. All the metal ions caused reduction in the activity of *C. lanatus* rind invertase at 50 mM concentration of each ion (Figure 7). In the presence of Fe^{2+}, there was an initial 19% increase in activity of *C. lanatus* rind invertase at 30 mM concentration, but a 48% reduction in activity occurred at 50 mM concentration when compared to control. In the presence of Cu^{2+}, there was also an initial 18% increase in the enzyme's activity at 20 mM concentration, while there was a 24% reduction in activity at 50 mM concentration when compared with control. In the presence of Co^{2+}, there was a 38% increase in activity at 20 mM concentration, while it brought about a 1% reduction in activity at 50 mM concentration when compared with control. However, in the presence of Zn^{2+} at 20 mM and 50 mM concentrations, there was a 29% and 9% increase in activity of the enzyme respectively, when compared with the control. It therefore means there is a certain concentration at which each of these metal ions will have beneficial effect on the activity of invertase after which, it becomes inhibitory to invertase activity.

Figure 8 shows the graphical representation of the effect of Zn^{2+}, Co^{2+} and Ag^{+} on the activity of *C. lanatus* rind invertase. The result obtained was compared with the control and it showed that the presence of Zn^{2+} caused considerably little or no effect on the activity of *C. lanatus* rind invertase; Co^{2+} was shown to cause a 17% increase in activity of the enzyme, while an 85% decrease in activity was observed in the presence of Ag^{+}.

Figure 9 shows a bar chart representing the summary of the effect of metal ion on the activity of *C. lanatus* rind invertase in comparison with the activity of the enzyme without any metal ion.

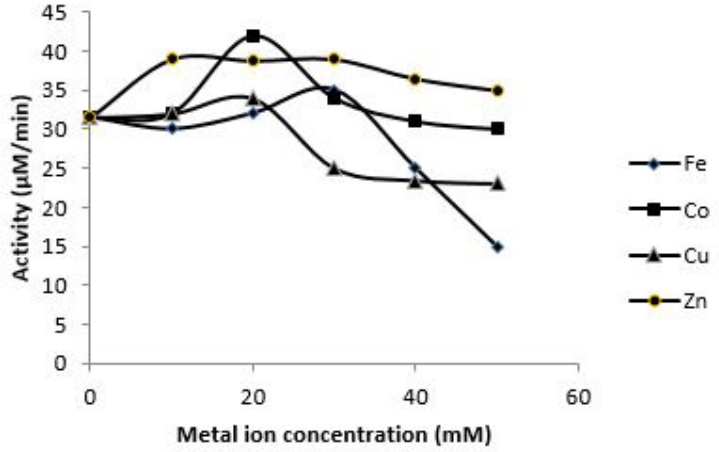

Figure 7: The effect of increasing concentrations of metal ions on *C. lanatus* rind invertase activity.

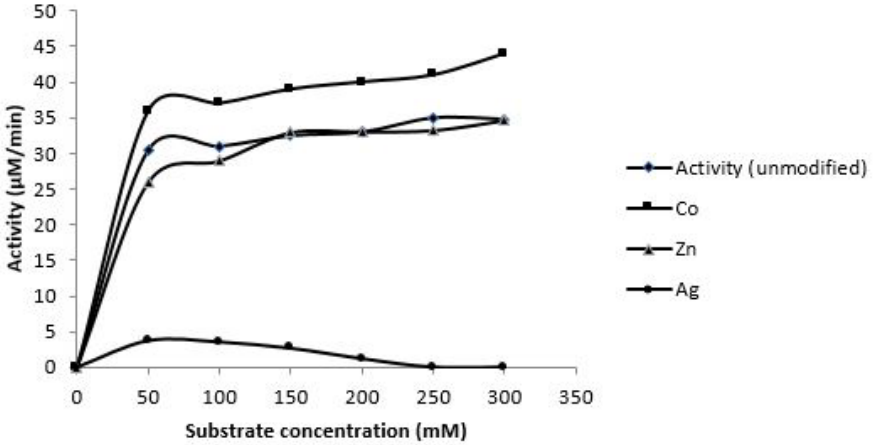

Figure 8: Activity of *C.lanatus* rind invertase in the presence and absence of Co^{2+}, $Zn^{2+}$and Ag^{+}.

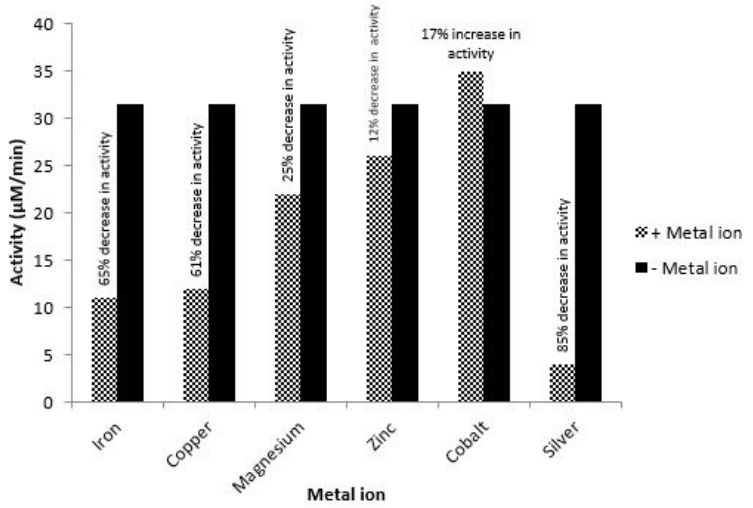

Figure 9: Summary of effect of metal ions on the activity of *C. lanatus* rind invertase.

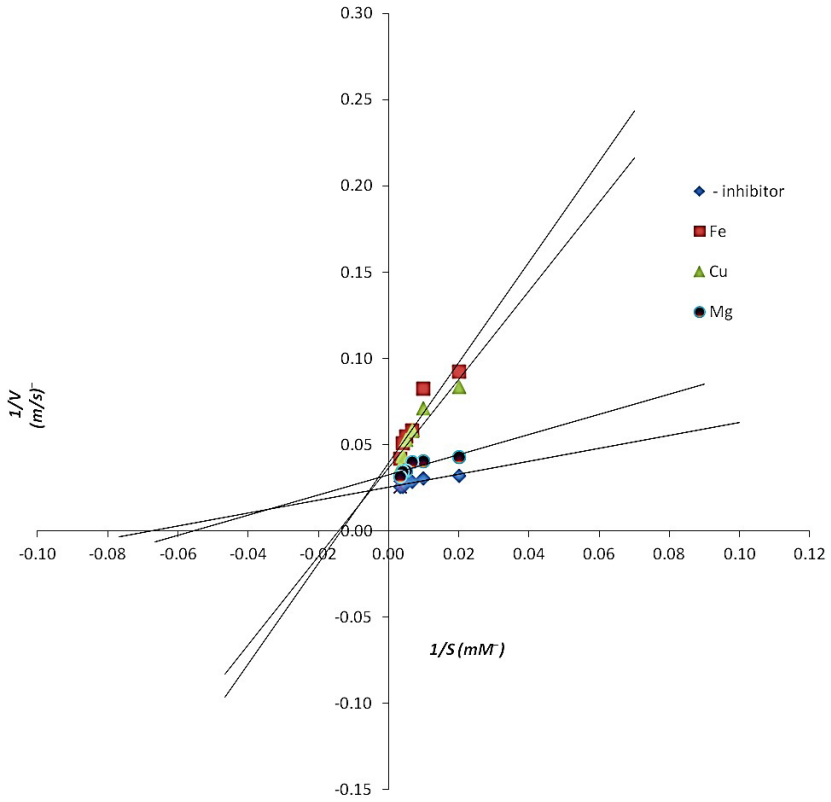

Figure 10: Double reciprocal plot for *C. lanatus* rind invertase activity in the presence of Fe^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+}.

Inhibition Studies

The mode of inhibition exhibited by Fe^{2+}, Cu^{2+} and Mg^{2+} was determined. Lineweaver-Burk plots were used to ascertain the mode of inhibition of *C. lanatus* rind invertase by the above listed metals. Figure 10 shows that all the investigated inhibitors exhibited the mixed type of inhibition.

DISCUSSION

The purification fold of 46.938 with a yield of 23% obtained from this study was higher than what was obtained for *Aspergillus terreus* which was 8.21 fold but with a better yield of 76.04% (Shaker, 2015). Similar to this observation is the work of Guimaraes *et al.* (2009) that reported 24% yield of purified invertase. Aslam *et al.* (2013) also reported a purification fold of 15 with recovery of 38 % yield for an extracellular invertase purified with ammonium sulphate precipitation and DEAE Sepahdex A-50.

The effect of pH on the activity of invertase isolated from *C. lanatus* rind was investigated. Stability was observed over a pH range of 2-9 with different buffers (acetate, phosphate, Tris-HCl) with the highest peaks at pH 4 and pH 7, which is an indication of the presence of acidic and alkaline invertases. This agrees with the result of Liu *et al.* (2005) who reported the presence of acidic and alkaline

invertase at pH 4.5 and pH 7 respectively in *Bambusa edulis*. Ionization state of amino acid residues present in the active site of an enzyme is normally pH reliant, so changes in the pH will significantly affect the ionic state of amino and carboxylic acid groups on the protein and consequently, the conformation and catalytic site of the enzyme (Essel and Osei, 2014).

Invertase enzyme from *C. lanatus* rind had an optimum temperature of 50 ºC. Similarly, invertase from *Saccharomyces cerevisiae* also showed highest activity at 50ºC, while that from *Saccharomycopis fibuligera* showed an optimum temperature of 55 ºC (Skowronek *et al.,* 2003). Biological reactions happen faster with increase in temperature until the point of enzyme denaturation, above which the enzyme activity and the rate of the reaction decreases abruptly (Marepally, 2017). The initial increase in enzyme activity as the temperature increases is possibly due to increase in reaction rate, as a result of increased kinetic energy of the reacting molecules.

Thermostability studies of *C. lanatus* rind invertase revealed that the enzyme was highly thermostable with optimum activity obtained at 50 ºC and 95 ºC after 20 mins of incubation. Esawy *et al.* (2014) reported that free invertase isolated from honey lost its activity completely at 70 °C after 45 minutes, while the immobilized enzyme kept 80% of its original activity at the same conditions. This suggest that it is very possible that immobilization of *C. lanatus* rind invertase will substantially increase its thermostabilty. Thermal stability is also an important criterion in choosing an enzyme for industrial use (Esawy *et al.,* 2014).

A double reciprocal plot of the enzyme affinity for sucrose gave a straight-line graph from which the Km was calculated to be 15 mM and the Vmax as 40 µM/min. The kinetic parameters are similar to that of Hsiao *et al.* (2002) who reported a Km of 15.28 mM for an invertase isolated from rice. However, Gallagher and Pollock (1998) reported a Km of 18 mM for *Lolium temulentum* invertase which was higher than the Km obtained in this study.

The observed decrease in activity of *C. lanatus* rind invertase in the presence of Fe^{2+}, Cu^{2+} and Mg^{2+} is similar to that reported by Esawy *et al.* (2014) in which Fe^{2+}, Cu^{2+} and Mg^{2+} brought about a reduction in the activity of invertase isolated from honey. Cu^{2+} also reportedly inhibited the invertase activities of carrot peels (Zill-e-Huma, 2015), and spent yeast (Kumar and Kesavapillai, 2015). Zn^{2+} was found to have relatively little or no effect on the activity of the enzyme, whereas Uma *et al.* (2010) reported Zn^{2+} to be a competitive inhibitor of the invertase enzyme. Kumar and Kesavapillai (2015) also reported slight inhibition of invertase activity by Zn^{2+}.

The activity of *C. lanatus* rind invertase was found to be enhanced by incubating it with Co^{2+}. The increase in activity of invertase in the presence of Co^{2+} is in correlation with many previous reports (Rubio *et al.,* 2002; Kumar and Kesavapillai, 2015; Zill-e-Huma, 2015). However, the activity of the enzyme was almost completely lost as 85% reduction in activity was observed on incubation with Ag^{+}. Inhibition or activation of invertase activity by metals may be due to the effect metals have on the amino acid residues present at the active site and the exterior surface of the enzymes. This perhaps may bring about alterations in the charge of the amino acids or structure distortions (Salis *et al.,* 2007).

CONCLUSION

This study established the presence of invertase activity in *Citrullus lanatus* rind. Invertase isolated from *C. lanatus* rind was found to have maximum activities at pH 4 and pH 7 and maximum temperature of 50ºC. It was found to be highly thermostable as it maintained high level of activity at 50ºC and 95ºC after incubation for 20 minutes. The activity of the enzyme was affected by the presence of metal ions in various degrees. Hence, *C. lanatus* rind may be recommended as a local source for the production of invertase enzyme, thus reducing the cost of production significantly.

DECLARATION OF CONFLICT OF INTEREST

The Authors declare that there is no conflict of Interest.

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