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Contributed Paper

An Orphan Crop (*Cucumeropsis mannii* Naudin) with Interesting Phosphatase, β-galactosidase and α-mannosidase Activities: Nutritional Approach and Biochemical Characterization

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ABSTRACT

Seeds extract of *Cucumeropsis mannii* Naudin (Cucurbitaceae), an orphan and under-researched crop was screened for enzymatic hydrolytic activities over a variety of natural and synthetic substrates. Enzymatic activities noticed mainly consist of phosphatase (0.66±0.02 UI/mg), β-galactosidase (0.26±0.03 UI/mg) and α-mannosidase (0.19±0.02 UI/mg) activities. The higher phosphatase activity observed suggest that this edible seed constitutes a potential source of phosphorus for human and animal diet. Exploration of biochemical properties has revealed that phosphatase, β-galactosidase and α-mannosidase activities were maximal at 45°C (pH 5.6), 55°C (pH 4.6) and 60°C (pH 5.0), respectively. The three enzymatic activities displayed also interesting characteristics as relative pH stability, resistance to (or activation by) detergents and hydrolytic specificity over natural substrates such as ATP, ADP, lactose and mannobioses. So, these disclosed enzymatic activities should deserve main investigations for further biotechnological applications.

Keywords: Orphan crop, *Cucumeropsis mannii* seed, cucurbitaceae, phosphatase, β-galactosidase, α-mannosidase.

1. INTRODUCTION

The usage of plant resources for various biotechnological purposes as food industry, genetic and medicine is of economic importance, social value and essential for humanity survival. In this respect, it is imperative to collect and conserve representative stocks of emerging plants with improved properties. It was from this perspective that neglected and underutilized crops also called minor or orphan crops [1, 2] such as those of the cucurbits family, have attracted attention for scientific research.

Cucurbitaceae (Cucurbits) is a plant family

commonly known as melons or gourds and includes crops like cucumbers, squashes (including pumpkins), luffas, and watermelons. The family is predominantly distributed around the tropics, where those with edible-seeded were among the earliest cultivated plants in both the Old and New Worlds [3]. These under-researched crops contribute to the diet of a large scale portion of resource-poor consumers and at the same time generate income for small-holder farmers in developing countries, particularly in Africa [4]. Thus, it is a major family for economically important species and some have nutritional, medicinal and other useable properties [5, 6]. In addition, they perform better than major crops of the world under extreme soil and climatic conditions [4].

Cucumeropsis mannii Naudin, also known as Mann's Cucumeropsis and white-seed melon in English, is a member of the indigenous edible cucurbits [2]. This neglected and underutilized vegetable has, at present, aroused attention due to its agronomic and economic potentials. Indeed, it has become material for production improvement and can be used in food conserve [4]. These interesting potentialities have resulted from this cucurbit's scientific interest that had consisted in its extensive characterization.

In Côte d'Ivoire, five species of cucurbits including *Cucumeropsis mannii* are very prized for their oleaginous seeds consumed as traditional soup thickeners locally called *pistachio* soup, and also called *egusi* soup in Cameroon, Nigeria and Benin [7]. This species of cucurbits is particularly reported to be highly rich in nutrients [8], namely protein (36±2.17%) and fat (45.89±4.73%) and is thus becoming more and more studied.

The genetic, nutritional and agronomic potentials of *C mannii* have been previously reported [6, 9-11], but the enzymatic potential of their seeds has not been yet explored. However, it is currently used as enzymes in biotechnological process [12-14]. So, the present work is conducing to highlight some enzymatic contents of *Cucumeropsis mannii* seeds with the aim of discussing their nutritional functions and then, biochemically characterizing the best ones for further biotechnological applications.

2. MATERIALS AND METHODS

2.1 Enzymatic Source and Enzymes Extraction

C. mannii seeds were obtained from the collection of the University of Abobo-Adjamé (Abidjan, Côte d'Ivoire). To obtain sufficient number of seeds, *C. mannii* was grown during its appropriate cropping season in 2008 at the experimental farm of the University ($5^{o}23$ latitude North, $4^{o}00$ longitude West, and 7 m altitude). After harvest, the fruits were split using a stainless steel machete, and then seeds were treated for the crude extract preparation. Cucurbit seeds (10 g) were ground using a blender in 20 ml sodium chloride solution 0.9% (w/v). The homogenate was subjected to sonication using a TRANSSONIC T_{420} for 10 min, and then centrifuged at 10,000 g for 30 min. The supernatant filtered through cotton was used as the crude extract (seeds extract).

2.2 Chemicals

para-nitrophenyl- (*p*NP-) glycopyranoside substrates (*p*NP-α-D-mannopyranoside (*p*NP-α-D-Man), *p*NP-α-D-glucopyranoside (*p*NP-α-D-Glu), *p*NP-β-D-glucopyranoside (*p*NP-β-D-Glu), *p*NP-α-D-galactopyranoside (*p*NP-α-D-Gal), *p*NP-β-D-galactopyranoside (*p*NP-β-D-Gal), *p*NP-α-L-fucopyranoside (*p*NP-α-L-Fuc), *p*NP-α-L-arabinopyranoside (*p*NP-α-L-Ara), *p*NP-β-D-xylopyranoside (*p*NP-β-D-Xyl)), *para*-nitrophenyl phosphate (*p*NPP), phenyl phosphate, sodium pyrophosphate, glucose-1-phosphate, glucose-6-

phosphate, fructose-6-phosphate, fructose-1-phosphate, α-nicotinamide adenosine dinucleotide (α-NAD), adenosine-2-3'-cyclo-monophosphate, adenosine-5'-triphosphate (ATP), phytate, *para*-nitrophenol (*p*NP), sucrose, starch, carboxymethylcellulose (CMC), Inulin, xylan, lactose, 2-O-α-D-mannopyranosyl-D-mannopyranoside (α-1,2 Mannobiose), 3-O-α-D-mannopyranosyl-D-mannopyranoside (α-1,3 Mannobiose), 6-O-α-D-mannopyranosyl-D-mannopyranoside (α-1,6 Mannobiose), D-mannose and D-glucose were purchased from Sigma Aldrich. Bovine serum albumin (BSA) was from Fluka Biochemika.

2.3 Hydrolytic Screening of Synthetic and Natural Substrates by Seeds Extract

For synthetic *p*NP-glycoside and *p*NP-phosphate hydrolytic activities, the crude extract was mixed in a total volume of 250 µl composed of the aryl-glycoside or aryl-phosphate substrate (5 mM, starting concentration) in 100 mM sodium acetate buffer pH 5.0 and the crude extract (50 µl which correspond to 0.3 mg of protein). The reaction mixture was incubated with shaking at 37°C for 10 min. The liberated *para*-nitrophenol (*p*NP) was quantified spectrophotometrically at 410 nm under alkaline conditions (2% w/v, Na_2CO_3) referred to a standard *p*NP (absorbance as a function of concentration) curve obtained in similar conditions. For natural substrates hydrolysis, the total volume was 300 µl, composed of 50 µl of the crude extract and 125 µl of substrate (0.25% w/v, final concentration) in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated with shaking at 37°C for 30 min, and the reactions were stopped by adding 150 µl of dinitro salicylic acid (DNS) and by heating the resulted solution at 100°C for 5 min [15]. The liberated reducing sugars were quantified spectrophotometrically at 540 nm referred to a standard glucose (absorbance as a function of concentration) curve obtained in similar conditions. All values were determined in triplicate. One unit of enzymatic activities in the two cases (synthetic and natural substrates) released 1 µmol of liberated product (*p*NP or reducing sugar) per min under the above conditions, respectively. The specific activity was expressed as µmol per min (UI) per mg of proteins.

2.4 Estimation of Protein Concentration

The concentration of the proteins was measured using the Folin ciocalteu method [16]. Bovine serum albumin (BSA) was used as the standard protein.

2.5 pH and Temperature Optima

The effect of pH on the enzymatic activities was determined by performing the hydrolysis of *para*-nitrophenylphosphate, *para*-nitrophenyl-β-D-galactopyranoside and *para*-nitrophenyl-α-D-Mannopyranoside in a series of buffers (100 mM) at various pH values ranging from 3.0 to 6.2. The buffers used were sodium acetate buffer from pH 3.6 to 5.5 and sodium citrate buffer from pH 3.0 to 6.2. The pH values of each buffer were determined at 25°C. The effect of temperature on phosphatase, β-galactosidase and α mannosidase activities was performed in 100 mM acetate buffer (appropriate pHs) over a temperature range of 30 to 80°C by using *p*NPP, *p*NP-β-D-Gal or *p*NP-α-D-Man (5 mM) as substrates under the enzyme assay conditions.

2.6 pH and Temperature Stabilities

The pH stability of each enzymatic activity was studied in a pH range of 3.0 to 6.2 with 100 mM sodium citrate buffer. The buffer was the same as that in pH and temperature optima study (above). After 2 h preincubation at 25°C, residual activities were measured at

37°C for 10 min by adding substrates (*p*NPP, *p*NP-β-D-Gal and *p*NP-α-D-Man). The thermal inactivation was determined at 37°C and at each enzymatic activity optimum temperature. Enzymes in appropriate buffers (pHs) were exposed to each temperature for the time range of 0-100 min. Aliquots were withdrawn at intervals and immediately cooled. Concerning thermal denaturation tests, aliquots of the crude extract were preheated at different temperatures ranging from 30 to 80°C for 15 min. Residual activities, determined in the three cases at 37°C under the enzymes assay conditions, were expressed as percentage activity of zero-time control of untreated enzymes.

2.7 Effect of Some Chemical Agents

To determine the effect of various compounds (cations, chelating, sulphidryl specific and reducing agents and detergents) as possible activators or inhibitors of the enzymatic activities, the crude extract was preincubated at 37°C for 30 min with the compounds and then, the activity was assayed under the enzymes assay conditions. The residual activities were expressed as percentage refers to a control without chemical agents.

2.8 Characterization of Hydrolytic Specificity

The hydrolytic specificity was determined by incubating the fraction of the seed extract (50 µl which correspond to 0.3 mg of proteins) with appropriate substrates for different times at 37°C in 100 mM sodium acetate buffer (each optimum pH). For phosphorylated substrates hydrolytic specificity, the inorganic phosphate (P_i) produced by the phosphatase activity was quantified by the method of Heinonen and Lahti [17]. Glucose and galactose released from the hydrolysis of lactose (1 mM) and mannose released from differently linked mannobioses (1 mM) were visualized through TLC plates. 3 µl Samples were spotted for each mixture. TLC plates were run with butanol-acetic acid-water (9:3.75:2.25, v/v/v), and then developed with naphto-resorcinol in ethanol and H_2SO_4 20% (v/v). The sugar spots were visualized at 110°C for 5 min.

3. RESULTS

3.1 Screening of Glycosidase and Phosphatase Hydrolytic Activities

The potential of synthetic and natural derivatives to act as substrates for the seed extract of *Cucumeropsis mannii* was assayed (Figure 1). We noticed that the crude extract hydrolyzed eleven (11) of the fourteen (14) substrates assayed. The best hydrolytic activities observed were phosphatase activity (0.66± 0.02 UI/mg) followed by β-D-galactosidase (0.26±0.03 UI/mg) and α-D-mannosidase (0.19±0.02 UI/mg) activities. There are apparently no α-D-glucosidase, α-D-fucosidase and β-D-xylosidase activities (Figure 1).

3.2 pH and Temperature Dependences

Phosphatase, β-galactosidase and α-mannosidase activities were analyzed for their hydrolytic pH and temperature dependences. The three enzyme activities were maximal at 45, 55 and 60°C and at pHs 5.6, 4.6 and 5.0, respectively (Figures 2A and 3). They retained 80% of their activity in sodium acetate buffer at a pH range of 4.0 to 6.2 (Figure 2B). The thermal stability study has revealed that at 37°C, the three activities remained stable for 100 min (Figure 4A). However, at their optimum temperature, these activities were less stable showing half-life (50% of activity) at least at about 60 min (Figure 4A). Phosphatase, β-galactosidase and α-mannosidase conserved 100% of their hydrolytic activity to temperatures up to their optima. Above, their activities declined progressively as the temperature

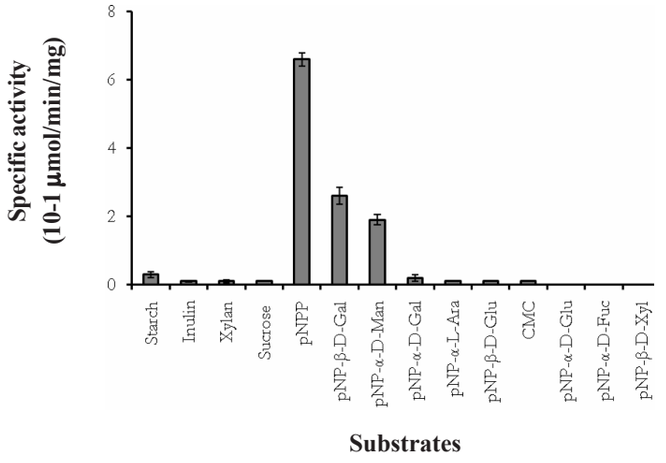

Figure 1. Screening of the seed extract from *cucumeropsis mannii* for several glycosidase and phosphatase activities over synthetic and natural substrates.

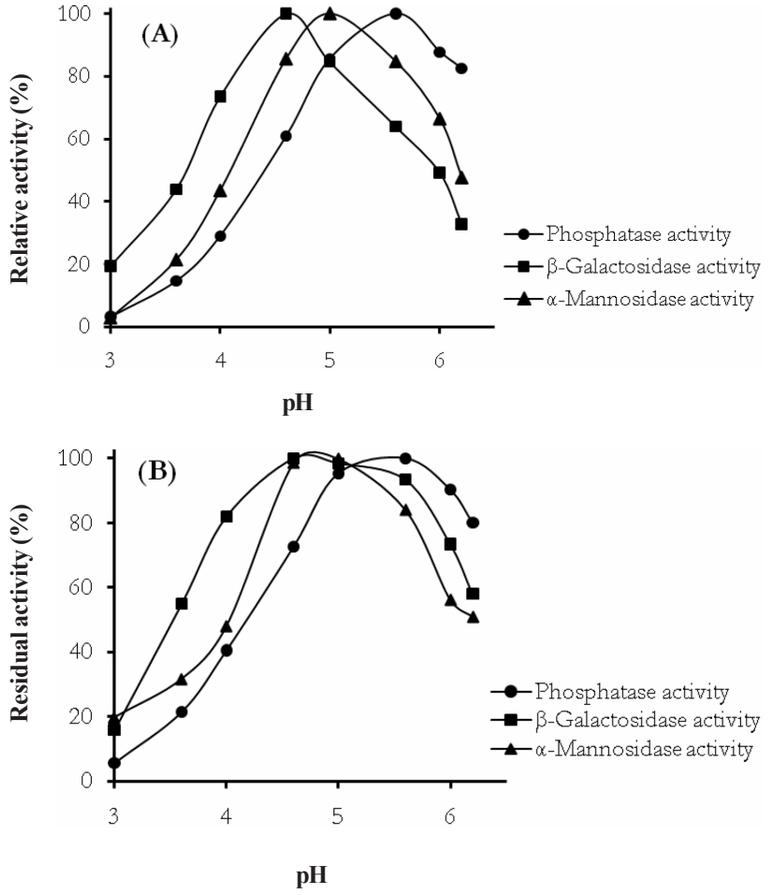

Figure 2. Effect of pH on phosphatase, β-galactosidase and α-mannosidase activities from the seed extract of *Cucumeropsis mannii*. (A) pH optimum, (B) pH stability.

increased (Figure 4B). Values of temperature coefficients (Q_{10}) calculated for the three enzyme activities were found to be for around 2; 1.8 and 1.5, respectively. From Arrhenius plot, values of 66.8, 49.2 and 30.2 kJ/mol were obtained for the activation energy of phosphatase, β-galactosidase and α-mannosidase activities, respectively (Data not shown).

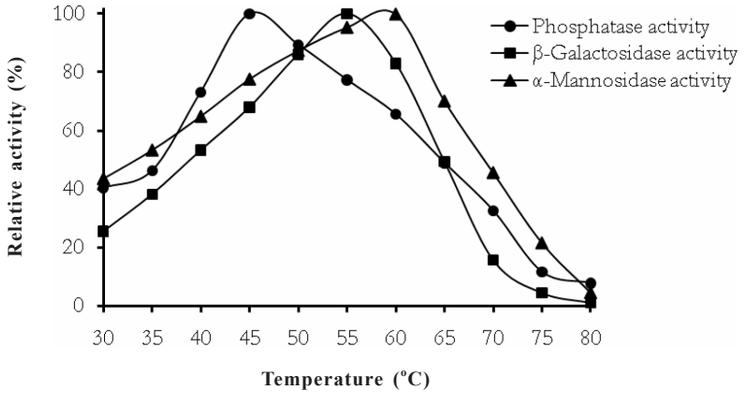

Figure 3. Effect of temperature on phosphatase, β-galactosidase and α-mannosidase activities from the seed extract of *Cucumeropsis mannii*.

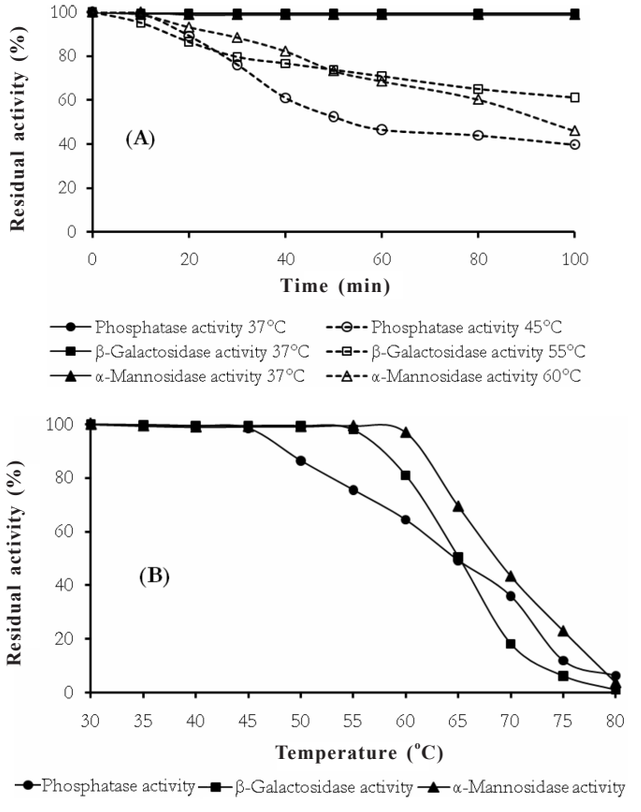

Figure 4. Thermal stability of phosphatase, β-galactosidase and α-mannosidase activities from the seed extract of *Cucumeropsis mannii*. (A) Thermal inactivation, (B) Thermal denaturation.

3.3 Effect of Cations, Chelating, Sulphidryl Specific and Reducing Agents

The effect of several chemicals on phosphatase, β-galactosidase and α-mannosidase activities by using the appropriate substrate was studied (Figures 5 and 6). All chemicals tested (Na^+, Ca^{2+}, Ba^{2+}, K^+, Mg^{2+}, EDTA, urea, *p*CMB, L-cysteine and β-mercaptoethanol) were found to be without significant effect on phosphatase activity (residual activity in the range of 93.5±2.1 to 109.2±3.5%). As concerned β-galactosidase activity, it was slightly inhibited for around 23% by *p*CMB (5 mM). However, this activity was enhanced in a range of 113±2.1 to 126.7±2.6% by 5 mM or 1% final concentration of Ca^{2+}, Ba^{2+}, EDTA, L-cysteine and β-mercaptoethanol (Figures 5 and 6). For α-mannosidase activity, most chemicals had no effect. Only Ba^{2+}, EDTA and urea have shown to be relatively inhibitory (between 22 and 42% of inhibition) (Figure 5).

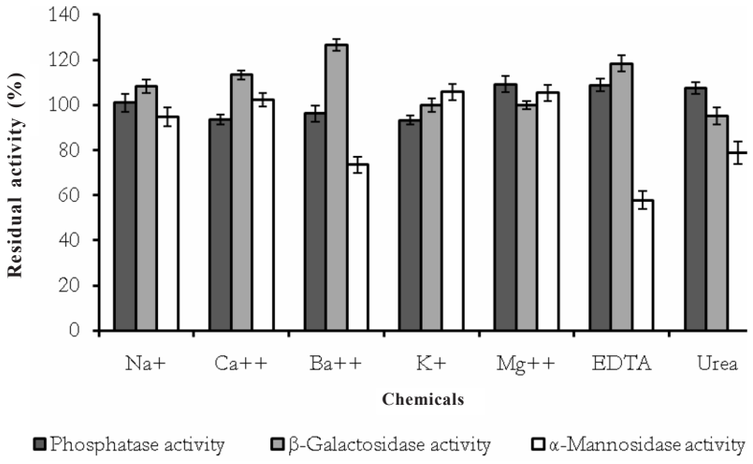

Figure 5. Effect of cations, chelating agent and urea on phosphatase, β galactosidase and α mannosidase activities from the seed extract of *Cucumeropsis mannii*.

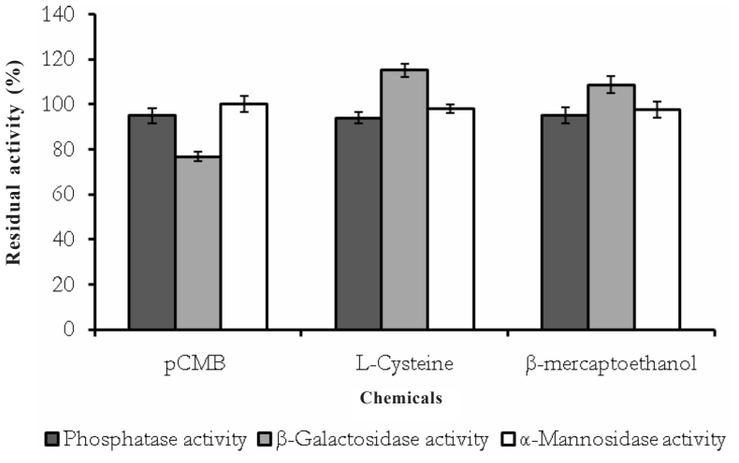

Figure 6. Effect *p*CMB, L-cysteine and â-mercaptoethanol on phosphatase β-galactosidase and α-mannosidase activities from the seed extract of *Cucumeropsis mannii*.

3.4 Effect of Detergents

Cationic, non-ionic and anionic detergents currently used for protein denaturing have shown, by and large, no significant effect on the three enzyme activities. However, tetradecyl trimethyl ammonium bromide, hexadecyl trimethyl ammonium bromide, polyoxyethylene-9-lauryl ether and polyxyethylene-10-tridecyl ether were found to enhance for up to 152±5.1% β-galactosidase activity while sodium dodecyl sulphate displayed a strong inhibitory effect (for around 96% inhibition) on phosphatase, β-galactosidase and α-mannosidase activities (Table 1).

Table 1. Effect of the detergents on phosphatase, β-galactosidase and α-mannosidase activities from the seed extract of *cucumeropsis mannii*.

Detergents*	Relative activity (%)		
	Phosphatase	β-Galactosidase	α-Mannosidase
Control	**100**	**100**	**100**
Cationic			
Tetradecyl Trimethyl Ammonium Bromide	75.5±4.8	152.0±5.7	85.7±3.6
Hexadecyl Trimethyl Ammonium Bromide	80.9±3.7	143.0±5.2	86.1±4.3
Non-ionic			
Tween 80	92.4±4.6	94.0±4.3	91.4±5.1
Lubrol Wx	107.4±3.8	100.0±2.4	93.1±2.5
Triton X-100	101.1±5.6	108.0±3.3	94.3±5.3
Anionic			
Polyoxyethylene-9-lauryl ether	107.4±4.3	122.0±5.2	93.0±4.3
Polyxyethylene-10-tridecyl ether	100.0±2.2	119.0±4.5	102.2±5.3
Polyxyethylene-10-oleyl ether	105.3±2.5	102.0±5.3	84.4±2.5
Sodium dodecyl sulphate	11.6±4.4	41.7±3.9	5.3±1.9

* = Assays were performed at 37°C for 10 min with 1% (w/v) starting concentration of detergent.

3.5 Substrate Hydrolytic Specificity

Phosphatase, β-galactosidase and α-mannosidase activities from seeds extract of *Cucumeropsis mannii* were assayed for their capabilities to hydrolyze different specific substrates (Table 2). The results showed that phosphatase activity hydrolyzed a broad range of phosphorylated substrates. The highest activity was observed with *p*-nitrophenyl phosphate (100±2.1%) followed by adenosine-5'-triphosphate (83.2±3.3%) and phenyl phosphate (72.5±2.0%). However, the hydrolysis of the two latter substrates remained interesting compared to that of sodium phytate (12.4±1.8%) and α-nicotinamide adenine dinucleotide (11.5±0.9%) which were less specific (Table 2). The ability of β-galactosidase activity from seeds extract of *C. mannii* to greatly hydrolyze lactose, a natural substrate, has been showed (Figure 7A). Thin layer chromatography (TLC) analysis of the hydrolysis of natural substrates namely α-1,2; α-1,3 and α-1,6 mannobioses by the α-mannosidase activity shows that, the three differently linked disaccharides were cleaved at different rates. α-mannosidase hydrolytic activity on α-1,2 mannobiose seemed to be greater than that on the two other linkages (Figure 7B).

Table 2. Hydrolytic specificity of phosphatase activity from the seed extract of *cucumeropsis mannii* over some phosphorylated substrates.

Substrates*	Hydrolytic activity (%)
p-Nitrophenyl phosphate	100.0±2.1
Adenosine-5'-triphosphate (ATP)	83.2±3.3
Phenyl phosphate	72.5±2.0
Sodium pyrophosphate	50.0±2.4
Glucose -6-phosphate	41.2±1.4
Fructose-6-phosphate	32.1±1.3
Glucose -1-phosphate	24.4±1.2
Fructose-1-phosphate	23.0±2.1
Adenosine-2'3'-cyclomonophosphate	19.8±3.2
Sodium phytate**	12.4±1.8
α-Nicotinamide adenine dinucleotide	11.5±0.9

* = Assays were performed at 37°C for 30 min with 5 mM final concentration of substrate, ** = This reaction was performed at 50C for 2 h.

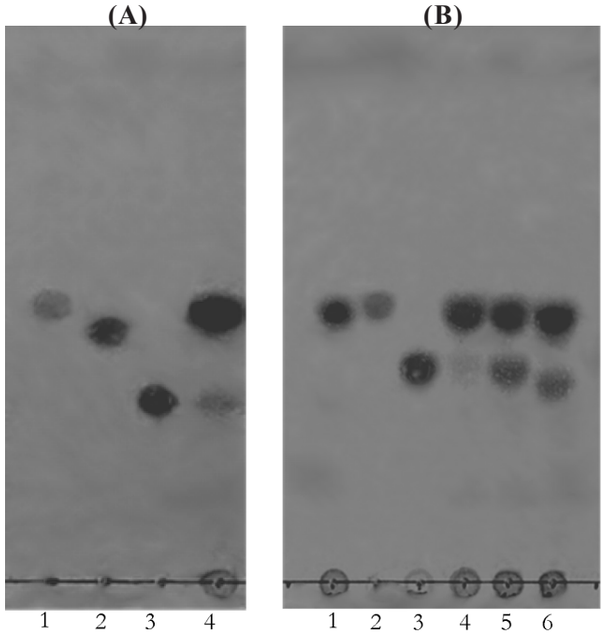

Figure 7. TLC plates showing β-galactosidase and α-mannosidase activities from the seed extract of *Cucumeropsis mannii* towards differently linked disaccharides. (A) β-galactosidase activity on lactose. Lane 1, glucose; Lane 2, galactose; Lane 3, lactose; Lane 4, reaction mixture. (B) α mannosidase activity on α-1,2; α-1,3 and α-1,6-mannobioses. Lane 1, crude extract; Lane 2, mannose; Lane 3, mannobiose; Lanes 4-6, reaction mixtures with α-1,2; α-1,3 and α-1,6-mannobiose, respectively.

4. DISCUSSION

Enzymes are essential biocatalysts to living organisms. Indeed, different aspects of biological functions have been resolved by studying physiological enzymes [18]. With the aim of diversifying sources of enzymes for further biotechnological application, the practical approach in enzymology to explore new biocatalysts, namely screening have been conduced. This study led to the assertion that *C. mannii* is an excellent source of enzymes, particularly an interesting phosphatase, β-galactosidase and α-mannosidase sources. Nevertheless, phosphatase activity was numerically large than the two latter.

Higher phosphatase activity is a good indicator of the present phosphorus status of a plant [19]. In this respect, it may be suggested that *C. mannii* seeds represent an important source of phosphorus for human and animal diet. However, the increase in phosphatase activity depended upon plant age, plant species and soil type [20]. Therefore, these affecting factors should also be taken into account for main investigations. Phosphatase, β-galactosidase and α-mannosidase specific activities noticed in the crude extract of *C. Mannii* seeds appeared to be higher than those already reported for plants and other sources, which were considered for purification [21-23]. So, this crop constitutes an interesting source of phosphatase, β-galactosidase or α-mannosidase which deserves be explored in great details for specific bio-industrial uses.

Biochemical characterization of the three enzymatic activities has revealed common and particular properties compared to those already reported. Indeed, pH and temperature studies have shown that these activities are acidic and mesophilic. This behaviour is in accordance with the majority of plant phosphatase, β-galactosidase and α-mannosidase activities already reported [21, 24, 25]. The three activities were conserved for around 80% in a pH range of 4.0 to 6.2 for 2 h. This residual stability remains suitable, and then constitutes a good compromise for performing hydrolysis or synthesis reactions for up to 2 h.

The behaviour of *C. mannii* seed extract phosphatase and β-galactosidase hydrolytic activities in the presence of divalent cations and cations chelator (EDTA) is tempting to speculate that the concerning ions were not strictly essential for the two enzymatic activities. However, the sensitivity of β-galactosidase hydrolytic activity to *p*CMB led to assume that -SH groups participate probably in this enzymatic reaction. As concerned α-mannosidase activity, the strong inhibitory effect caused by EDTA may suggest its metallo-dependence's as already shown in the case of *Drosophila melanogaster* α-mannosidase [26].

There is by and large, a tendency for the three enzymatic activities to remain stable or activated by most of detergents tested. This behaviour should constitute an advantageous characteristic for their potential bio-industrial usage. Furthermore, the strong stimulatory effect displayed by the cationic detergents tested on β-galactosidase activity make them particularly useful when extract this activity by improving its stability for further specific characterization.

Phosphatase activity from the seed extract of *C. mannii* was not strictly specific since it released inorganic phosphate from a variety of phosphorylated substrates. Although this activity was maximal toward the synthetic aryl substrate *p*-nitrophenyl phosphate, it also showed considerable activity other a natural substrate (ATP). This relatively high activity toward ATP indicates the possibility to apply this enzymatic activity to synchronous enzyme-reaction system, which needs energy resulting from the hydrolysis of this substrate [24]. As well as phosphatase activity, β-galactosidase

activity has also showed ability to greatly hydrolyze a natural substrate (lactose) and thus, could serve as an exo-glycosidase in releasing galactose from oligogalactosides. Furthermore, enzymatic hydrolysis of lactose has two main biotechnological applications: the utilization of whey as glucose and galactose (the hydrolysates) having greater fermentation potential [27] and in the production of low lactose milk (and dairy products made from it) for consumption by lactose intolerant persons. To date, the majority of industrially used β-galactosidase preparations were either from bacterial or fungal sources [28]. However, Dey [29] had reported that widely distributed plant β-galactosidase could be a good substitute for lactose hydrolysis in industries due to easy availability and cost effectiveness. In this respect, β-galactosidase from *C. Mannii* seeds could also be explored for industrial application. As for α-mannosidase activity, it displayed broad natural substrate specificity by hydrolyzing α-1,2; α-1,3 and α-1,6 mannobioses linkages. This behaviour bears out a close resemblance to α-mannosidases involved in the degradation of oligomannose type free N-glycans which exhibit biological activity as growth factor during the early development of plant [30] and stimulation of fruit ripening [22, 31]. For the specific application, the purified α-mannosidase(s) from the seeds extract of *Cucumeropsis mannii* could also find the importance in the combination with xylanase in pulp and paper industry as already showed by Viikari *et al.* [32].

5. Conclusion

To sum up this report, it may be conclude that phosphatase, β-galactosidase and α mannosidase activities from the seed extract of *Cucumeropsis mannii* displayed interesting nutritional and biochemical characteristics that deserve main investigations for further biotechnological applications.

Acknowledgements

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