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Characterization of Crude Thermostable Exoinulinase Produced by *Saccharomyces* sp.

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Abstract

Studies were carried out to investigate the properties of extracellular inulinase produced by Saccharomyces *sp. in solid state fermentation of wheat bran. The pattern of inulin hydrolysis by the crude inulinase enzyme as revealed by thin layer chromatography analysis of the products of hydrolysis showed the inulinase to be exo-acting. Temperature and pH studies revealed that the inulinase preparation had an optimum activity of 125.4 units per gram of dry substrate (U/gds) at 50ºC while an optimum inulinase activity of 128.3 U/gds was observed at pH 4.5. The crude inulinase preparation retained 82% of its activity at 50ºC after heating for two hours. Effect of various thermal stabilizers on the activity of the crude inulinase showed that glycerol had the best thermal stabilizing effect on the activity of the crude inulinase preparation. Potassium and calcium ions were found to enhance the activity of the enzyme while mercury and silver ions completely inhibited the inulinase activity. The crude inulinase preparation had a V_{max} and K_m values of 416 and 1.47 mM, respectively.*

Keywords: *Fermentation, wheat bran, temperature, pH, thermal stability. glycerol.*

Introduction

Inulin is a polydispersed fructan consisting mainly of β-(2→1)-D-fructosyl-fructose links terminated by a sucrose residue (De Leenheer 1996). It is reported to be the next abundant polysaccharides after starch (Kango and Jain 2011), found in many plants of compositae and gramineae and also accumulated in the underground roots and tubers of several plants including *Vernonia herbacea*, Jerusalem artichoke (*Helianthus tuberosus*), chicory (*Cichorium intibus, Cichorium endivia*), and dahlia (*Dahlia pinnata*) where it acts as storage polysaccharide (Gupta and Kaur 1997). Inulin from various sources are reported to vary in there degree of polymerization (Chi *et al*. 2011). Furthermore, the degree of polymerization is reported to be a function of the harvesting period, and storage time after harvesting (Chi *et al*. 2011). Inulinasesare fructofuranosyl hydrolases that act on the β-2,1 linkage of inulin, resulting in the formation of fructose, glucose and inulooligosaccharides depending on the pattern of action of inulinase (Onilude *et al*. 2012). The inulinases have become the cynosure of researchers primarily due to its application in the production of high fructose syrup (García-Aguirre *et al*. 2009; Mutanda *et al*. 2009; Ricca *et al*. 2009) and fructooligosaccharides (Yun *et al*. 2000; Mutanda *et al*. 2008; Risso e*t al*. 2012) via the hydrolysis of inulin. Inulinases have also been reported to be useful in the production of ethanol via the fermentation of the fructose produced during the hydrolysis of inulin (Nakamura *et al*. 1996; Zhang *et al*. 2010a and b), acetone and butanol, pullulan exopoly-saccharide, gluconic acid and sorbitol, and alsosingle-cell oil and single-cell protein production (Chi *et al*. 2011). Although inulinases from various organisms have been reported (Uzunova *et al*. 2001; Zherebtsov *et al*. 2002; Sharma *et al*. 2006; Singh *et al*. 2007; Yuan and Bai 2008; Mazutti *et al*. 2010), only a few of these organisms have been able to

produce sufficient inulinases which fulfil the desired characteristics of high temperature optimum and thermal stability (for the successful application of inulinases in the various industries)hence, the need to screen for as well isolate new inulinase-producing microorganism that can meet the various inulinase demand of the various inulinase utilizing industries. Onilude *et al*. (2012) reported inulinase production by a newly isolated *Saccharomyces* sp. in solid state fermentation using wheat bran as substrate. This paper reports the characteristic of the crude inulinase produced by this *Saccharomyces* sp. in solid state fermentation of wheat bran.

Materials and Methods

Microorganism

The inulinase-producing *Saccharomyces* sp. used in this study was obtained from the Culture Collection Centre of The Department of Microbiology University of Ibadan. The organism was previously isolated from spontaneously fermented sugar-cane (Onilude *et al*. 2012). It was sub-cultured on yeast extract-peptone-sucrose (YPS) agar medium containing (g/l) yeast extract 2.5, peptone 5.0, sucrose 15.0 and agar 20.0 incubated at 30°C for 48 hours.

Inoculum Preparation

Inoculum was prepared by transferring 1-ml cell suspension of 24 hold culture of the organism into a liquid medium (100 ml) containing (g/l) sucrose (20.0), yeast extract (5.0), K_2HPO_4 (5.0), NH_4Cl (1.5), KCl 1.15), and $MgSO_4 \cdot 7H2O$ (0.65), in an Erlenmeyer flask as described by Mazutti *et al*. (2006). Flasks were incubated at 35°C and 150 rpm for 24 hours and 2 ml of this was used as the inoculum.

Solid State Fermentation

Solid state fermentation was carried out as described by Onilude *et al*. (2012) using wheat bran as substrate. The wheat bran was inoculated with a 2-ml suspension of the inoculum above. The moisture content of the wheat bran was adjusted to 65% (w/v) while the pH was adjusted to 5.5. Incubation was done at 35ºC for 72 hours after which the inulinase produced was extracted as described by Mazutti *et al*. (2006).

Assay of Inulinase

Inulinase activity was determined according to Burkert *et al*. (2006). Crude enzyme extract (0.1 ml) was incubated at 50°C for 15 min with 0.9 ml of 0.1 M sodium acetate buffer (pH 5.5) containing 2% inulin. Thereafter, the enzyme was inactivated by keeping the reaction mixture at 90°C for 10 min. The reaction mixture was then assayed for glucose as a reducing sugar using the DNSA method (Miller 1959). Absorbance of the reaction mixture was measured using a Jenway Spectrophotometer at 540 nm. One unit of inulinase activity was defined as the amount of inulinase enzyme that produced 1 µmol fructose per minute under standard assay conditions.

Pattern of Inulin Hydrolysis

Pattern of hydrolysis of inulin (whether exo- or endo-acting) by the crude inulinase extract was determined by spotting the reaction product (after incubating inulin and the crude inulinase for 2 hours at 50^{o}C) on a pre-coated TLC plate (Merck Germany). The plate was developed with a solvent system of chloroform: acetic acid: water (30:35:5 v/v/v) at room temperature and sugars were visualized by heating the plate at 120^{o}C for 10 min after which it was sprayed with 1% α-naphtol (containing 10% phosphoric acid) acid as described by Azhari *et al*. (1989).

Determination of Optimum pH and pH Stability of the Crude Inulinase

The effect of pH on the activity of the crude enzyme was determined by incubating 0.1 ml of the crude enzyme with 0.9 ml of inulin substrate preparation at pHs between 3.0-7.0 using 0.1 M sodium acetate for pH 3.0-5.5, citrate phosphate buffer for pH 6.0-6.5, sodium phosphate buffer for pH 6.5-7.0 and thereafter measuring the reducing sugar liberated as stated above (Chen *et al*. 2009). The pH stability of the enzyme was also studied by incubating the crude enzyme at

different pHs followed by incubation with inulin substrate and assaying for reducing sugar liberated afterwards.

Determination of Optimum Temperature and Thermostability of the Crude Inulinase

The optimum temperature for the activity of the crude inulinase was determined by incubating 0.1 ml of the enzyme with 0.9 ml of the substrate mixture at different temperature ranges (40, 45, 50, 55, 60, 65 and 70°C) for 15 min at pH 4.5 and the liberated reducing sugar measured as described above. Thermal stability of the enzyme was also studied by incubating the crude enzyme at the various temperatures (40, 50, 60 and 70ºC) for 2 hours and assaying for the residual inulinase activity afterwards.

Effect of Thermal Stabilizers on the Crude Enzyme Activity

The effect of various thermal stabilizers on the activity of the enzyme was done as described by Gill *et al*. (2006) by incubating the enzyme with 10% w/v of stabilizers (ethylene glycol, sorbitol, glycerol, dextran) at 70ºC for 3 hours. Then the residual activity was estimated by incubating 0.1 ml of the recovered enzyme in each experiment with 0.9 ml of 2% inulin substrate in 0.1 M sodium acetate buffer at pH 5.5 and the amount of released reducing sugars was estimated. In all experiments, each set up was replicated thrice.

Effect of Various Metal Ions and Other Chemicals on the Activity of the Inulinase

The effect of various metal ions on inulinase activity was investigated by incubating 0.1 ml of the crude enzyme solution with 0.9 ml of the inulin containing 2 mM of K^+, Fe^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Al^{3+}, Cu^{2+}, Zn^{2+} and Ag^+ in 0.5 M sodium phosphate buffer (pH 7.0) at 50 °C for 30 min, then assaying for residual enzyme activity.

Also, the effects of 2 mM EDTA and 10 mM urea on inulinase activity were determined by incubating 0.1 ml of the crude enzyme solution with 0.9 ml of inulin containing EDTA and urea at 50 °C for 30 min, then assaying for the residual enzyme activity as previously stated.

Kinetic Parameters of the Crude Inulinase

For the investigation of the kinetic parameters of the crude inulinase preparation, 0.4 U/ml of the crude inulinase preparation was incubated with the inulin concentration ranging from 0.2×10^{-4} to 4.0×10^{-4} M as described by Zang *et al*. (2004) and the inulinase activity measured for each of the substrate concentrations as described earlier. The K_m and V_{max} values were determined by the method of Lineweaver-Burk.

Results and Discussion

The results of the pattern of inulin hydrolysis by the crude inulinase preparation from *Saccharomyces* sp. is shown in Fig. 1. Exo-acting inulinase releases fructose as the main product of hydrolysis while endo-acting inulinases release mixture of fructose and other inulooligosaccharides (Kango and Jain 2011). The absence of any inulooligosaccharides in the reaction mixture suggests that the inulinase preparation from this *Saccharomyces* sp. is exo-acting. Similar inulinase preparations that are exo-acting have been isolated and purified from various inulinase-producing organisms such as: *Kluyveromyces marxianus* strain CBS 6556 (Rouwenhorst *et al*. 1998), *Chrysosporium pannorum* AHU 9700 (Xiao *et al*. 1989); *Aspergillus fumigatus* (Gill *et al*. 2006); *Penicillium* sp. TN-88 (Moriyama *et al*. 2002); and *Aspergillus awomari* (Arand *et al*. 2002). The exo-acting nature of this inulinase makes it of potential importance in the food industries where it can be used in the production of ultra high fructose syrup.

The results of the effect of pH on activity of the crude inulinase preparation presented in Fig. 2 showed that the optimum pH was 4.5 with an activity of 128.3 U/gds while the lowest activity of 35.3 U/gds was observed at pH 7.0. Similar values were found in the literature. *Kluyveromyces*sp. Y-85 maximum inulinase activity was observed at pH 4.5 (Wenling *et al*. 1999) while with *K. marxianus* DMS 70106, Pessoa and Vitolo (1999) described a high inulinase activity between pHs 3.2 and 5.0.

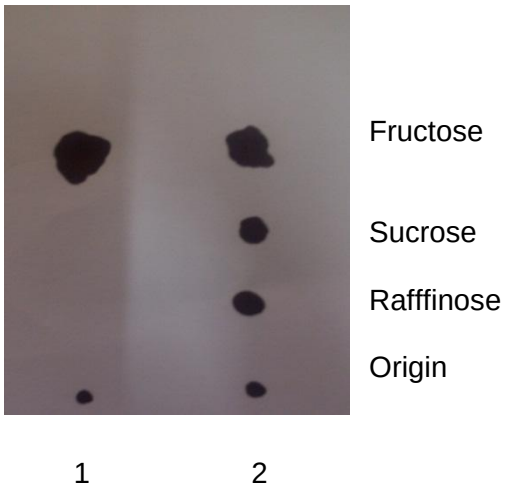

Fig. 1. TLC analysis showing the pattern of hydrolysis of inulin by the crude inulinase by *Saccharomyces* sp. (Lane 1); sugar standards (Lane 2).

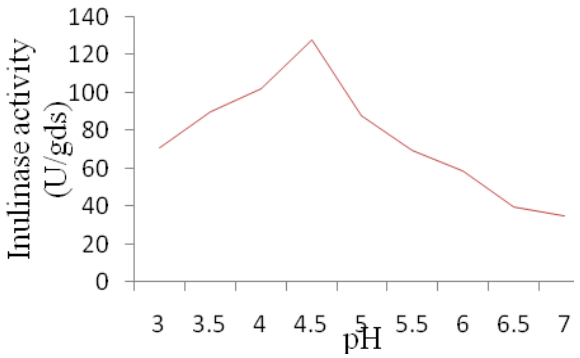

Fig. 2. Effect of different pH values on the activity of inulinase produced by *Saccharomyces* sp. Data are presented as mean ± SEM, *n* = 3.

Kushi *et al*. (2000) and Ettalibi and Baratti (2001) obtained maximum inulinase activity at pH 4.7 for *K. marxianus* var. *bulgaricus* and *Aspergillus ficcum*, respectively. Loss of inulinase activity beyond the optimum pH could be as a result of the changes in the state of acidic or basic amino acids in the protein. Changes in pH may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis.

At 50°C, the crude inulinase preparation was stable over a wide range of pH (4.0-7.0) with the enzyme activity drastically reduced outside this range and the enzyme almost lost its activity beyond 3.0 and 9.0 (data not shown). The results of optimum temperature determination and thermal stability of the crude inulinase are presented in Figs. 3 and 4. The data showed that the crude inulinase preparation from *Saccharomyces* sp. had an optimum temperature of 50°C. Optimum activity at 50°C has been reported for inulinase preparations from various organisms by Workman and Day (1983) and Cruz-Guerrero *et al*. (1995). High inulinase activity at relatively elevated temperatures is an interesting factor from the industrial point of view considering the limited solubility of inulin at room temperatures (Ettalibi and Baratti 2001; Gill *et al*. 2006). The crude enzyme retained 82% (106.49 U/gds) of its activity after 2 hours of heat treatment (at 50°C), 69% (88.53 U/gds) (at 60°C) and 30% (38.49 U/gds) at (70°C) while there was complete inactivation of inulinase activity at 80°C.A good thermal stability at 50°C but not at 60°C for the strain *K. marxianus* CDBB-L-278 was also described by Cruz-Guerrero *et al*. (1995).

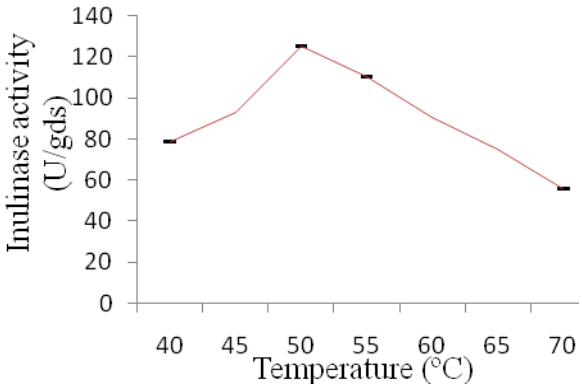

Fig. 3. Effect of different temperature values on the activity of inulinase produced by *Saccharomyces* sp. Data are presented as mean ± SEM, *n* = 2.

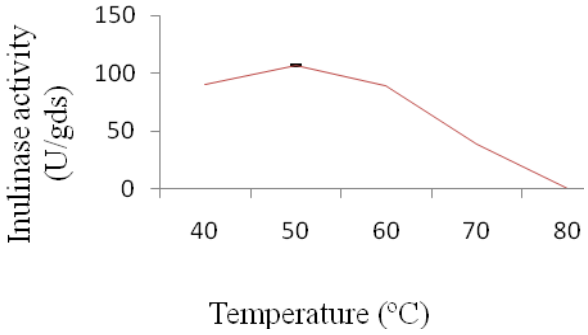

Fig. 4. Thermal stability of inulinase produced by *Saccharomyces* sp. Data are presented as means ± SEM, *n* = 3.

Similarly, Gill *et al*. (2006) reported 76% residual of activity of inulinase by an inulinase isoform of *Aspergillus fumigatus*. Higher temperature optimum and pH stability by the inulinase from *Saccharomyces* sp. could also be as a result of the fact that the enzyme was prepared in solid state fermentation (Pandey *et al*. 2000). Enzymes with higher optimal temperature of activity and stability at high temperatures are attractive for biotechnological application in various industrial sectors.

The results of the effects of various thermal stabilizers on the thermal stability of the crude inulinase (represented in Fig. 5) showed that glycerol had the best stabilizing effect with an activity of 106.01 U/gds as compared to 81.12 U/gds (control experiment). Similar results have been previously reported by Öngen-Baysal *et al*. (1994) and Taylor *et al*. (1995). Glycerol having the best stabilizing effect could be as a result of preferential exclusion of the polyols with proteins, which increases with an increasing polyol size (Liu *et al*. 2010), resulting in an indirect interactions that prevent the protein from thermal unfolding (Taravati *et al*. 2007).

The effect of various metal ions at 2 mM concentration on the inulinase activity of the extracellular inulinase from *Saccharomyces* sp. is presented in Table 1. A total loss of activity was observed in the presence of Hg^{+} and Ag^{+} while in the presence of K^{+} and Ca^{2+}, the inulinase activity increased from 100±0.08 U/gds to 116.8±0.41 U/gds and 104.6±4.20 U/gds, respectively. A reduced activity was also observed in the presence of Fe^{2+} (Table 1). Workman and Day (1983) reported that various metal ions affect the activity of enzymes by either inhibiting or stimulating enzyme activity. The complete inhibitory effect of Hg^{2+} observed in this study has also been reported earlier (Ettalibi and Baratti 1987; Kochhar *et al*. 1997; Sharma *et al*. 2006).

The inhibitory effect observed could be due to the presence some thio (-SH) group in the active site of the enzyme which is necessary for the catalytic activity of the inulinase. In this work, EDTA had no significant effect on enzyme activity. This suggests that the inulinase preparation is not dependent on divalent ion as they affect the catalytic activity of the enzyme as it relates to divalent cofactors (Guimarães *et al*. 2007). The effect of metal ions on enzyme activity may be relevant when considering the use of substrate with high salt content (Bhatti *et al*. 2006). The kinetic parameters, as investigated by a Line-weaver Burk plot, revealed that the crude inulinase had a K_m and K_{cat} of 2.86 mM and 1.71×10^{2} s^{-1}, respectively. High K_m and K_{cat} values by the inulinase preparation imply a high affinity for inulin substrate by the enzyme (Gill *et al*. 2006).

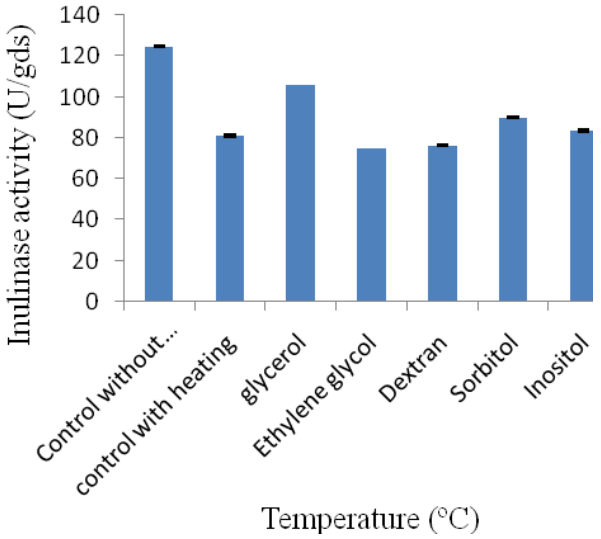

Fig. 5. Effect of different thermal stabilizers on inulinase activity produced by *Saccharomyces* sp. Data are presented as mean ± SEM, *n* = 3.

Table 1. Effect of various metal ions on inulinase activity by *Saccharomyces* sp.

Metal ions	Concentration (mM)	Inulinase activity (U/gds)
Control	-	119±0.08
Mg^{2+}	2	84.2±0.29
Cu^{2+}	2	44.2±0.49
Mn^{2+}	2	48.5±6.12
Ag^{+}	2	n.d.
K^{+}	2	126.8±0.41
Ca^{2+}	2	134.6±4.20
Fe^{2+}	2	n.d.
Zn^{2+}	2	86.3±2.83
Na^{+}	2	135.2±1.42
Hg^{2+}	2	n.d.
Urea	10	58.0±0.49
EDTA	2	122.5±0.09

Note: Data are presented as mean ± S.D; n.d. = not detected; and *n* = 3.

Conclusion

In conclusion, this work presents the characteristics of crude inulinase preparation from *Saccharomyces* sp. The pattern of inulin hydrolysis by this enzyme, the relatively high thermal stability and high activity at slightly acidic pH makes it of potential importance in the production of fructose from inulin. However, there is a need to further investigate the kinetics of inulin hydrolysis by this crude inulinase preparation in order to develop an appropriate model the application of the enzyme in fructose production.

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