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Effect of extrinsic and intrinsic parameters on inulinase production by *Aspergillus niger* ATCC 20611

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Abstract

Background: Inulinase is a versatile enzyme from glycoside hydrolase family which targets the β-2, 1 linkage of fructopolymers. In the present study, the effect of medium composition and culture conditions on inulinase production by *Aspergillus niger* ATCC 20611 was investigated in shake-flasks. **Results:** The highest extracellular inulinase (3199 U/ ml) was obtained in the presence of 25% (w/v) sucrose, 0.5% (w/v) meat extract, 1.5% (w/v) $NaNO_3$ and 2.5 mM (v/v) Zn^{2+}, at initial pH of 6.5, temperature 35ºC and 6% (v/v) of spores suspension in the agitation speed of 100 rpm. Surfactants showed an inhibitory effect on enzyme production. The optimum temperature for inulinase activity was found to be 50ºC. TLC analysis showed the presence of both exo- and endo-inulinase. **Conclusion:** Sucrose, Zn^{2+}, and aeration were found to be the most effective elements in inulinase production by *A. niger* ATCC 20611. TLC analysis also showed that the crude enzyme contained both endo and exo-inulinases. The strain is suggested as a potential candidate for industrial enzymatic production of fructose from inulin.

Keywords: *Aspergillus niger* ATCC 20611, culture optimization, inulinase

INTRODUCTION

Inulinase (2, 1-β-D-fructan fructanohydrolase, EC3.2.1.7) is a versatile enzyme from glycoside hydrolase family (fructanohydrolases), which targets the β-2, 1 linkage of fructopolymers like inulin (Dilipkumar et al. 2011). Generally, inulinase from microbial sources can be separated into classes, endo- and exo-inulinase. Endoinulinase splits off the inulin with an endocleavage action, that breaks down internal β-2,1 fructofuranosidic linkages to produce a chain of fructooligosaccharides such as pentaose, tetraose and inulotriose. On the other hand, exoinulinase degrades the terminal fructose units from inulin, raffinose and sucrose sequentially (Kulminskaya et al. 2003; Kango, 2008; Saber and El-Naggar, 2009).

Although inulinase was initially isolated from plants, it is difficult to obtain high production (Kumar et al. 2005). In the last decades, a large number of microorganisms such as bacteria (*Clostridium* sp.,

Xanthomonas sp., *Bifidobacterium* sp., *Geobacillus* sp., *Bacillus* sp., *Thermotoga* sp., *Pseudomonas* sp.), yeast (*Kluyveromyces* sp.) and filamentous fungi (*Penicillium* sp., *Fusarium* sp., *Aspergillus* sp.) were used for inulinase production (Souza-Motta et al. 2005; Kango, 2008; Singh and Bhermi, 2008; Naidoo et al. 2009). Inulinase has a wide range of applications: for high fructose syrup obtaining from inulin, increase iron absorption in children, to improve calcium absorption in menopausal women, to increase ethanol removal from blood of highly intoxicated persons (alcoholics), to produce of alcohol, acetone, gluconic acid, sorbitol, pullulan and production of inulo-oligosaccharides, and to use as a prebiotic for improving population of beneficial microorganisms such as *Bifidobacterium* in intestinal flora (Souza-Motta et al. 2005; Naidoo et al. 2009; Saber and El-Naggar, 2009).

The present study was conducted to optimize medium composition and culture conditions for biomass and inulinase production by *A. niger* ATCC 20611.

MATERIALS AND METHODS

Microorganism and preparation of inoculums

Aspergillus niger ATCC 20611 was used as inulinase producing strain. The strain was cultured on potato dextrose agar (PDA) and stored at 4-8ºC for routine use throughout the experiments. The spores were harvested and suspended in sterile distilled water containing 0.01% (v/v) Tween 80 to obtain a concentration of 2.0 x 10^6 spores/ml.

Shake-flask fermentation

Initial experiments were performed by using a basal medium containing 1% (w/v) inulin and 0.5% (w/v) peptone as recommended by Singh et al. (2007). The initial pH of the medium was adjusted at 6.5 using 5 M NaOH or 5 M HCl prior to sterilization at 121ºC for 15 min. The strain was grown in the basal medium at 30ºC in a shaker incubator (150 rpm) for 4 days. All the experiments were carried out in triplicates in 250 ml Erlenmeyer flasks containing 50 ml of the medium inoculated with 6% (v/v) of the inoculums.

Experimental design

Medium optimization. In a preliminary study, the growth profile of *A. niger* ATCC 20611 and inulinase production was investigated for 7 days in the basal medium. Influence of nutritional and physical factors on growth and inulinase production were examined by using one-factor-at-a-time design. The effect of various carbon sources was studied using 1% (w/v) of different carbon sources including glucose, fructose, sucrose, maltose and fructan.

The effect of various nitrogen sources ($NH_4H_2PO_4$, $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3,KNO_3, beef extract, yeast extract; urea, malt extract and meat extract) was also tested at a final concentration of 0.5% (w/v), in the present of sucrose (1% w/v) as carbon source. To determine the best concentration of nitrogen source (inorganic and organic), further optimization was carried out using different concentrations of $NH_4H_2PO_4$ and meat extract from 0.5 to 5% and 0.05 to 5% (w/v), respectively. Different combinations of inorganic nitrogen sources with 0.5% (w/v) of meat extract were also tested.

Enzyme production was also assessed in the presence of various metal ions including Ca^{2+}, Zn^{2+}, k^{+}, Al^{3+}, Mg^{2+} and Na^{+} at the concentration of 0.5 mM. Further optimization was carried out to determine the best concentration of Zn^{2+}. The effect of various surfactants (0.5%) including Brij-35, sodium dodecyl sulphate (SDS), Tween 20, Tween 60, Tween 80 and Triton X-100 was also investigated. All the experiments were carried out at pH 6.5 and 30ºC in a shaker incubator with agitation speed of 150 rpm for 96 hrs. Inulinase activity, biomass and pH were tested at 12-hrs intervals.

Process parameters optimization. In 250 ml Erlenmeyer flasks containing 50 ml of the optimal medium containing 1% (w/v) sucrose, 0.5% (w/v) meat extract, 1.5% (w/v) $NaNO_3$ and 2.5 mM (v/v) Zn^{2+}, the effect of culture conditions on inulinase production and biomass was studied. The effect of initial pH on inulinase production was tested by growing the strain at different pH values ranging from 4

to 12. The effect of temperature was examined by incubating the inoculated flasks (pH 6.5) at different temperatures ranging from 20 to 50ºC for 96 hrs. Inulinase production was also studied at 35ºC and pH 6.5 for 96 hrs in different inoculum sizes from 2% to 12% (v/v) and different agitation of 0-250 rpm. All the experiments were carried out in triplicate and the values were reported as mean ± standard deviation.

Characterization of the crude enzyme and hydrolysis products. Enzyme activity was evaluated at different temperatures ranging from 30ºC to 60ºC. Thin layer chromatography (TLC) was used for qualitative analysis of the reaction products. Pre-coated TLC plates (silica gel 60 plate) spotted with samples were developed using chloroform:acetic acid:water (30:35:5, v/v/v) as irrigating solvent. The hydrolysis products (sugars) were visualized by heating the plates at 120ºC for 30 min after spraying with detection solution containing 0.1 g α-naphthol and phosphoric acid 10% in absolute ethanol.

Analytical method for inulinase activity. The culture was centrifuged at 10,000 x *g* for 20 min at 4ºC, and the supernatant was used to measure inulinase activity by spectrometric determination of reducing sugars, as previously described by Miller (1959). Briefly, 0.5 ml of the supernatant was incubated with 0.5 ml of 1% (w/v) inulin (substrate) in sodium acetate buffer (200 mM, pH 5.0) at 50ºC for 20 min; the amount of the reducing sugars was determined by DNS reagent. Enzyme activity (U/ml) was defined as the amount of enzyme required to liberate 1 µmol of fructose per min under the assay conditions using fructose as the calibration standard. Biomass was determined by dry weight measurement. Biomass, pH, and extracellular inulinase were tested every 12 hrs for 7 days. All the assays were carried out in triplicate.

Statistical analysis. Data analysis was carried out using one-way analysis of variance (one-way ANOVA) and Duncan's Multiple Range test by the Statistical Package for the Social Sciences SPSS. Statistical significance was set at $P < 0.05$ and the results were expressed as means ± standard error of mean.

RESULTS AND DISCUSSION

Growth curve and inulinase production by *A. niger* ATCC 20611

A. niger ATCC 20611 was grown in the basal medium; growth rate, inulinase production and pH were measured at different time points. As shown in Figure 1, the maximum inulinase production and growth rate of 319 U/ml and 2.5 mg/ml respectively, were obtained after 96 hrs of incubation at 30ºC in a shaker incubator with agitation speed of 150 rpm. Inulinase production was shown to be coincided with the exponential growth phase.

The pH was found to be decreased from 6.5 to 3.0 during the assay; maximum inulinase activity was observed at pH 4.6 after 96 hrs of incubation (Figure 1). This was in agreement with the previous studies on *A. tamarii* (Kango, 2008) and *A. niger* (Saber and El-Naggar, 2009), where during inulinase production a decrease in pH values from 5.5 to 3.5 and 6.0 to 4.5 after 72 hrs and 96 hrs, respectively was reported. This may be due to the deamination of some amino acids or the formation of organic acids (Souza-Motta et al. 2005). According to the previous studies by Jing and Augustine (Jing et al. 2003) and Naveen (Kango, 2008), in optimal condition, *A. ficuum* JNSP5-06, after 120 hrs, and *A. niger* NK-126, after 96 hrs, were able to produce 25 U/ml and 52.5 U/ml of inulinase, respectively. The observed decline in inulinase activity after 96 hrs of incubation could be a result of protease degradation, decrease in nutrient availability in the medium and catabolic repression of the enzyme (Wang and Zhou, 2006; Kango, 2008).

Effect of carbon sources

Inulinase has been shown to transfer a fructosyl moiety to a terminal 2-β-fructofuranoside at the primary hydroxyl group; the donor and acceptor of the fructosyl moiety could be sucrose or other inulin-type sugars (Rubio and Navarro, 2006). Inulinase retention by cell wall and secreted from cells resides mainly in the cell wall, where the diffused sucrose can be easily hydrolyzed. Such specific localization of inulinase may be ecologically beneficial for the efficient scavenging of hydrolyzed products. However, this may not be the case for the other carbon sources because other sugars molecules can

hardly penetrate into the cell wall and must therefore be hydrolyzed outside the cell wall (Lertwattanasakul et al. 2011). All the carbon sources used in this study were found to support growth, however, the highest activity of inulinase (347 U/ml) was found to be in the presence of sucrose (Table 1). Sucrose has been also reported as the best inducer for inulinase production by *Kluyveromyces marxianus* YS-1 (Singh et al. 2007). The present study also investigated the optimum concentration of sucrose for enzyme production; maximum inulinase yield (928 U/ml) was obtained with 25% (w/v) of sucrose, as also reported in the previous studies (Wang and Zhou, 2006; Santos et al. 2007). Moreover, biomass production was increased up to 25 mg/ml by increasing the concentration of sucrose up to 15% (w/v).

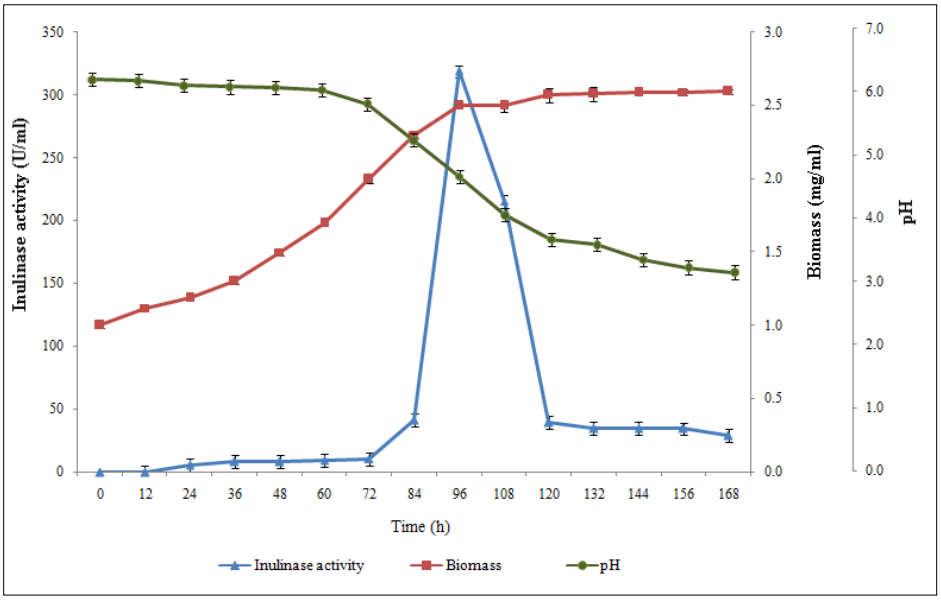

Fig. 1 Time course study of inulinase production by *A. niger* ATCC 20611. The fungus was grown in 50 ml basal medium at 30ºC with agitation at 150 rpm. Values are presented as mean ± S.D of triple determinations.

Decline in inulinase production at high sucrose concentrations (>25%) and at the presence of other carbon sources could be due to catabolic repression of the enzyme synthesis or secretion of proteolytic enzymes, which are known to cause enzyme denaturation (Singh et al. 2007). This may also be attributed to carbon source limitation at the end of the fermentation process (Ariff and Webb, 1998).

Effect of inorganic nitrogen sources

As shown in Table 1, maximum production of inulinase (359 U/ml) was observed in the control medium (without inorganic nitrogen sources). In addition, a decrease in inulinase production was recorded in the culture medium supplemented with 0.5 and 1% of $NH_4H_2PO_4$. By increasing the concentration of $NH_4H_2PO_4$ up to 2%, pH decreased from 6.5 to 3.5 and the production of inulinase was totally repressed (data not shown). Suppressive effect, even at very low concentration of $NH_4H_2PO_4$, may due to pH decline. Acidic pH (less than 4.0) can possibly account for the loss of enzyme activity (Singh et al. 2007).

Effect of organic nitrogen sources

The maximum inulinase activity of 606 and 525 U/ml was obtained in the culture medium containing meat extract and yeast extract, respectively (Table 1). Of the various concentrations of meat extract (0.05-5%, w/v), maximum inulinase activity was observed at 0.5% (w/v). An increase in biomass (19.7 mg/ml) was observed by increasing the meat extract concentration up to 4% (w/v) (data not shown). Meat extract has been also reported as suitable nitrogen source for *Kluyveromyces marxianus* YS-1

(Singh et al. 2007). However, tryptone, beef extract and corn steep liquor (CSL) have been reported to be favourable organic nitrogen sources for inulinase production by *A. niger* (Kango, 2008), *Xanthomonas campestris pv. phaseoli* KM 24 mutant (Naidoo et al. 2009), *A. officinalis* (Singh and Bhermi, 2008) and *A. tamari* (Saber and El-Naggar, 2009), respectively.

Inulinase activity was shown to be increase up to 628 U/ml by increasing the meat extract concentration up to 0.5% (w/v); however, in higher concentrations it was declined. This could be due to the toxic effects of its constituents, at high concentration, on inulinase activity (Singh et al. 2007).

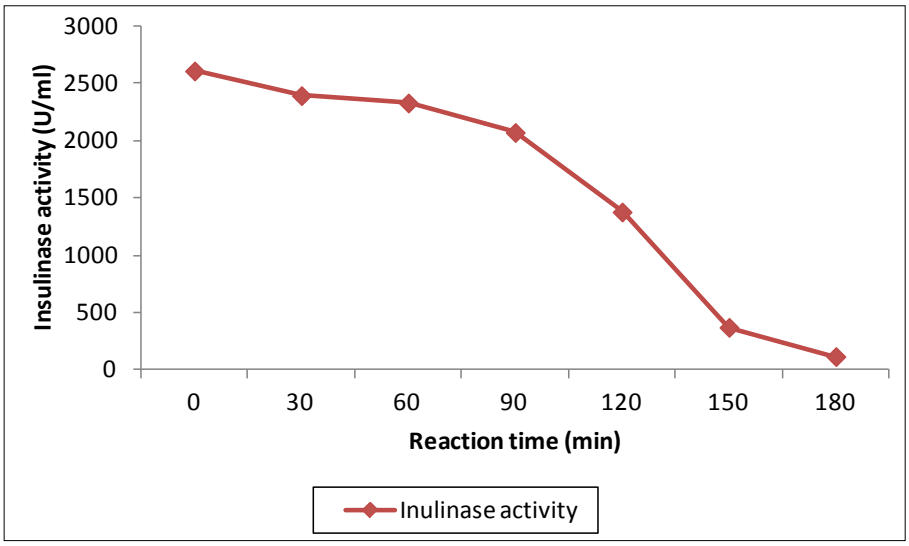

Fig. 2 Thermo stability of crude inulinase by *A. niger* ATCC 20611. Inulinase activity was determined by using inulin as the substrate with incubation at optimum temperature 50ºC for 180 min. Values are mean ± S.D of triple determinations.

Effect of combining organic and inorganic nutrient sources

A mix of nitrogen sources is thought to be more effective for enzyme production and growth compared to inorganic and organic nitrogen sources when applied individually (Singh and Bhermi, 2008). The preset study revealed that the combination of meat extract and nitrates can significantly ($p \leq 0.05$) increase inulinase production by *A. niger* (Table 1), as also reported by Gill et al. (2003) for *Streptomyces sp.* GNDU 1. Liberation of free acids by using ammonium ions is thought to inhibit metabolic process by causing acidic conditions in the medium (Singh and Gill, 2006).

Optimum inulinase production (811 U/ml) was obtained at the presence of 1.5% (w/v) $NaNO_3$ and 0.5% (w/v) meat extract; however, a decrease in enzyme production was detected by increasing the amount of $NaNO_3$ up to 5%. Increase in enzyme production may due to increase in fungal growth; on the other hand, the observed decrease in high concentration of $NaNO_3$ could be due to the complex nature of $NaNO_3$, as its constituents, at higher concentration, might have toxic effects on enzyme production or inhibit the secretion of inulinase (Skowronek and Fiedurek, 2004).

Effect of metal ions

Among the metal ions, Zn^{2+} was found to be more effective in inulinase production (Table 1). Post-transition metal ions like Al^{3+} were also shown to stimulate the growth of *A. niger*, although they were unable to support inulinase production. Maximum inulinase production (1466 U/ml) and fungal growth (38 mg/ml) was detected in the presence of 2.5 mM (w/v) Zn^{2+}. The same stimulation effect was reported for *A. ficuum* (Jing et al. 2003), *A. fumigatus* (Gouda, 2002) and *Kluyveromyces marxianus* YS-1 (Singh et al. 2007) in the presence of Zn^{2+}, Ca^{2+} and Mn^{2+}, respectively. This effect may due to the formation of complex with ionized inulinase resulting in changing solubility and behaviour at the

substrate interfaces. In addition, during the fermentation, transition metal ions may change the conformation of protein to a less stable form by interaction with enzyme surface charge which could markedly affect the ionization of some amino acid residues (Masomian et al. 2010).

Effect of surfactants

All the surfactants used in this experiment were found to repress inulinase (Table 1). A rapid decrease in inulinase production from 1281 U/ml to 288 U/ml was recorded in the present of 0.01 to 2.5% of Triton X100 (data not shown). Surfactants change the permeability of cell membrane leading to easy release of enzymes into the medium (Costas et al. 2004).

Effect of pH

The initial pH was found to impact inulinase production. The maximum production of inulinase (1519 U/ml) and biomass (41 mg/ml) was observed at pH 6.5 in potassium phosphate buffer, while enzyme production was shown to be very low at acidic (pH 4.0) and alkaline (pH 12) conditions. The pH 6.5 has been also recommended for optimal inulinase production by *Kluyveromyces marxianus* YS-1 (Singh and Bhermi, 2008) and *A. niger* AUP19 (Kumar et al. 2005). *Xanthomonas campestris pv. phaseoli* KM 24 mutan, *A. tamari, Cryptococcus aureus* G7 and *A. fumigates* have shown to produce maximum inulinase at pH 7.0, 5.5, 5.0 and 6.0, respectively (Gouda, 2002; Sheng et al. 2007; Naidoo et al. 2009; Saber and El-Naggar, 2009).

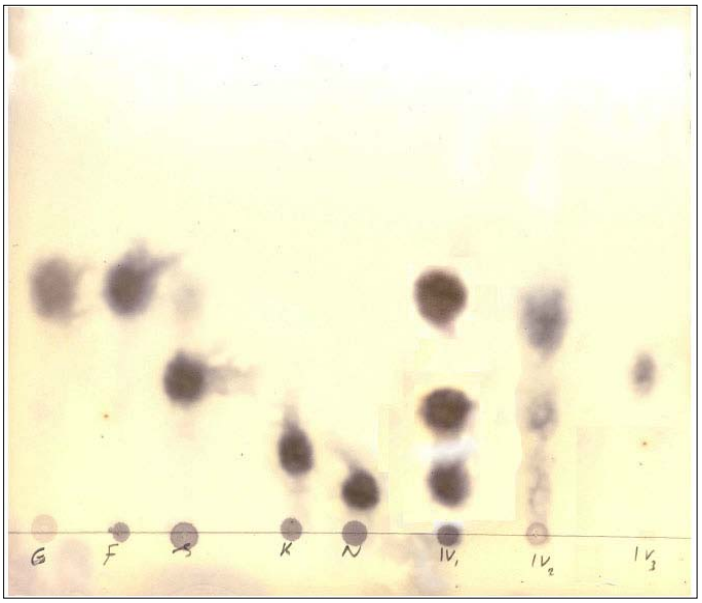

Fig. 3 Thin layer chromatographic analysis of inulin hydrolysis by inulinase from *A. niger* ATCC 20611. G: glucose, F: fructose, S: sucrose, K: ketose, N: nystose, Iv_1: optimization medium (with 25% of sucrose), Iv_2: optimization medium (with 1% of sucrose), Iv_3: basal medium the end products of inulin hydrolysis was analyzed by using TLC plate (silica gel 60 plate) with the solvent system of chloroform: acetic acid: water (30:35:5, v/v/v). Basal medium culture filtrate was incubated with pure inulin (1% w/v) solution in 0.1M sodium acetate buffer pH 5.0 at 50ºC for 60 min. detection reagent comprising 0.1 g α-naphthol and phosphoric acid10% in absolute ethanol.

Effect of temperature

Different temperatures have been reported for inulinase production; for example, 30ºC for *A. fumigates* (Gouda, 2002) and *Kluyveromyces marxianus* YS-1 (Singh and Gill, 2006), and 28ºC for *Cryptococcus aureus* G7 (Sheng et al. 2007) and *A. niger* AUP19 (Kumar et al. 2005). As shown in Table 1, 35ºC was found to be the optimum temperature for inulinase production (2472 U/ml) by *A. niger* ATCC 20611. A decrease in enzyme production was observed above or below of this temperature, as it was

also reported in the previous studies (Naidoo et al. 2009; Saber and El-Naggar, 2009). Low inulinase production at higher temperature could be due to the reduction of oxygen solubility in the medium, or enzyme denaturation (Masomian et al. 2010). In the case of extracellular enzymes, temperature may influence their secretion, possibly by changing the physical properties of the cell membrane (Ebrahimpour et al. 2011).

Effect of inoculum size (spore density)

Inoculum size plays an important role in fermentation process; in a suitable inoculum size, sufficient amount of nutrient and oxygen will be accessible for growth. Different optimum inoculum sizes have been reported for inulinase production, for example: 1% for *A. niveus Blochwitz* 4128 URM (Souza-Motta et al. 2005), 2% for *A. niger* (Skowronek and Fiedurek, 2004), and 5% for *A. tamarii* (Saber and El-Naggar, 2009). As shown in Table 1, 6% of 10 days old culture was found to be the most suitable inoculum size for inulinase and biomass production by *A. niger* used in this study; less enzyme activity was recorded above and below of this inoculum size. At high inoculum size, the viscosity of fermentation medium might increase due to the tremendous growth of fungi, resulting in nutritional imbalance in the medium or maybe using up the nutrients before they are physiologically ready to start enzyme production (Singh et al. 2007). Low inulinase production using 1% (v/v) inoculum may due to insufficient fungal biomass.

Effect of aeration

The results showed a progressive increase in inulinase activity and growth, when agitation speed was increased to 100 rpm (Table 1). Interestingly, Gouda (2002) reported that *A. fumigates* was able to produce high inulinase without any agitation. This may be due to the high values of specific oxygen uptake rate by the strain, resulting in the liberation of a proteolytic enzyme and hydrolysis of inulinase. However, in other studies, significant increase in inulinase production has been reported under agitation compared to static condition (Singh et al. 2006; Singh et al. 2007; Singh and Bhermi 2008). Agitation leads to better dispersion of substrate, nutrients and oxygen in medium (Park and Yun, 2001; Singh et al. 2007). High agitation may reduce fungal growth because during respiration hydrogen atoms may combine with oxygen, forming hydrogen peroxide, which is lethal to the cell (Masomian et al. 2010).

Characterization of activity, stability and inulin hydrolysis by crude inulinase

Inulinase activity was strongly affected by temperature (Table 1). The produced inulinase was shown to have optimal activity at 50ºC. Lower temperatures have reported for other microorganisms; for example 45ºC for *A. fumigatus* (Gouda, 2002), 37ºC for *A. niveus Blochwitz* 4128 URM (Souza-Motta et al. 2005), 46ºC for *Streptomyces* sp. GNDU1 (Gill et al. 2003) and 40ºC for *A. tamarii* (Saber and El-Naggar, 2009).

Thermostability was examined by incubating the crude enzyme at 50ºC in a water bath for 3 hrs. The enzyme retained 92% of its activity after 30 min; however it was reduced to 89%, 53% and 5% after 1, 2 and 3 hrs of incubation, respectively (Figure 2). The enzyme was found to be inactivated at temperatures more than 60ºC (data not shown), as also reported in the previous studies (Kumar et al. 2005; Kango, 2008; Singh and Bhermi, 2008). At high temperatures, the flexibility of enzyme is thought to be increased and therefore the enzyme may bind loosely to the substrate. Consequently, turnover number of enzyme is decreased, resulting in a gradual decline in enzyme activity (Masomian et al. 2010).

TLC analysis was used to determine the exo- or endo-nature of the crude inulinase. It was showed that monosaccharides and oligosaccharides were the predominant end and exo-product over a hydrolysis time of 1 hr (Figure 3). The end-products of inulin hydrolysis were shown to be monosaccharides and shorter inulooligosaccharides (Ohta et al. 2002; Sheng et al. 2007; Kango, 2008; Naidoo et al. 2009). In contrast, only exoinulinase activity has been reported for the produced inulinase by *Kluyveromyces marxianus* YS-1 and *Streptomyces* sp., liberating only fructose (Gill et al. 2003; Singh and Bhermi, 2008).

CONCLUDING REMARKS

A significant increase in inulinase production by *A. niger* ATCC 20611 was achieved by optimizing medium composition and process parameters at shake flask. In the optimal medium containing sucrose (25% w/v), meat extract (0.5% w/v), $NaNO_3$ (1.5% w/v) and 2.5 mM of Zn^{2+}, over 3199 U/ml of inulinase activity was recorded within 96 hrs of incubation at 35ºC, pH 6.5, 6% (v/v) inoculum size and agitation speed of 100 rpm. The produced inulinase showed the highest activity at 50ºC. The results also showed the liberation of a large amount of monosaccharides and oligosaccharides after inulin hydrolysis by the enzyme, indicating that the crude enzyme contained both endo and exo-inulinases.

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Table

Table.1 Effect of the culture conditions on inulinase and biomass production by *A. niger* ATCC 20611.

	Inulinase activity (U/ml)	Biomass (mg/ml)		Inulinase activity (U/ml)	Biomass (mg/ml)
Carbon sources (1%, w/v)			**Surfactants (0.5%, w/v)**		
Glucose	321 ± 2.5891[a]	2.7 ± 0.062[a]	Tween 20	0.00 ± 0.0000	9.0 ± 1.0709
Fructose	318 ± 2.5822	2.4 ± 0.063	Tween 60	1269 ± 5.0356	14 ± 1.0680
Sucrose	347 ± 2.6966	3.7 ± 0.064	Tween 80	246.0 ± 5.0360	11 ± 1.0300
Maltose	295 ± 2.7010	1.9 ± 0.062	Triton X 100	1363 ± 5.0318	23 ± 1.0410
Inulin	278 ± 2.5611	2.1 ± 0.063	SDS	28.00 ± 5.0143	7.0 ± 1.0572
Fructan	267 ± 2.8721	2.0 ± 0.061	Birj 35	1073 ± 5.0259	10 ± 1.0480
Control medium[b]	2.00 ± 2.6100	0.5 ± 0.006	Control medium[h]	1468 ± 5.2610	38.2 ± 1.086
Basal medium	298 ± 2.5611	2.1 ± 0.061	Basal medium	305.0 ± 2.5250	2.3 ± 0.1250
Inorganic nitrogen sources (0.5%, w/v)			**Inoculum size (%, v/v)**		
$(NH_4)_2SO_4$	0.00 ± 0.0000	3.6 ± 0.133	2	1656 ± 9.2329	32 ± 1.0838
$NaNO_3$	0.00 ± 0.0000	3.8 ± 0.131	4	2239 ± 9.2890	35 ± 1.0908
NH_4Cl	16.0 ± 2.6770	4.0 ± 0.132	6	2493 ± 9.2980	51 ± 1.0904
$NH_4H_2PO_4$	126 ± 2.5991	6.8 ± 0.133	8	2441 ± 9.2917	38 ± 1.0901
NH_4NO_3	117 ± 2.4900	5.2 ± 0.131	10	1757 ± 9.2212	36 ± 1.0902
KNO_3	0.00 ± 0.0000	3.0 ± 0.126	12	1233 ± 9.2873	27 ± 1.0901
Control medium[c]	359 ± 2.4697	4.0 ± 0.128	Control medium[i]	2493 ± 9.2981	51 ± 1.0901
Basal medium	306 ± 2.5611	2.4 ± 0.128	Basal medium	305.0 ± 2.5250	2.3 ± 0.1250
Organic nitrogen sources (0.5%, w/v)			**Agitation (rpm)**		
Meat extract	606 ± 4.4974	7.0 ± 0.135	0	194.0 ± 9.0422	45 ± 1.0014
Malt extract	309 ± 4.0079	5.3 ± 0.135	100	2561 ± 9.0547	61 ± 1.0013
Beef extract	450 ± 4.0057	7.6 ± 0.136	150	2500 ± 9.0515	54 ± 1.0015
Yeast extract	525 ± 4.0054	9.1 ± 0.134	200	1002 ± 9.0377	29 ± 1.0011
Urea	141 ± 4.0052	0.7 ± 0.134	250	773.0 ± 9.0241	25 ± 1.0010
Peptone	347 ± 4.0042	3.7 ± 0.135	Control medium[j]	2500 ± 9.0515	54 ± 1.0015

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Control medium[d]	347 ± 4.0042	3.7 ± 0.135	Basal medium	305.0 ± 2.5250	2.3 ± 0.1250
Basal medium	298 ± 4.0051	2.6± 0.132			
Meat extract (0.5%, w/v) and inorganic nitrogen sources (0.5%, w/v)			**pH**		
$(NH_4)_2SO_4$	172 ± 5.0128	5.4 ± 0.1483	Sodium acetate 4	0.000 ± 0.0000	4.1 ± 1.0340
$NaNO_3$	641 ± 5.0137	11± 0.1471	Sodium acetate 5	178.0 ± 5.7551	4.4 ± 1.0446
NH_4Cl	137 ± 5.0110	6.2 ± 0.1483	Sodium acetate 6	1260 ± 5.6768	37 ± 1.0273
$NH_4H_2PO_4$	223 ± 5.0149	7.6 ± 0.1500	Potassium phosphat 6	1423 ± 5.4740	37 ± 1.0644
NH_4NO_3	496 ± 5.0117	11± 0.1414	Potassium phosphat 6.5	1519 ± 5.7150	41 ± 1.0990
KNO_3	334 ± 5.0116	6.6 ± 0.1457	Potassium phosphat 7	1334 ± 5.8745	35 ± 1.0300
Control medium[e]	606 ± 4.4974	7.0 ± 0.1350	Potassium phosphat 8	1187 ± 5.8650	32 ± 1.1000
Basal medium	306 ± 2.5611	2.4 ± 0.1283	Tris-HCl 8	1131 ± 5.7190	8.6 ± 1.0000
Metal ions (0.5 mM, v/v)			Tris-HCl 9	920.0 ± 5.7390	30 ± 1.0300
Al^{3+}	689 ± 5.0127	23 ± 0.5013	Glycine 9	802.0 ± 5.6010	4.8 ± 1.0583
Zn^{2+}	956 ± 5.0122	20 ± 0.5013	Glycine 10	113.0 ± 5.4340	3.6 ± 1.0300
K^{+}	540 ± 5.0122	18 ± 0.5014	Glycine 11	33.00 ± 5.8730	2.4 ± 1.0400
Mg^{2+}	608 ± 5.0126	14 ± 0.5013	Disodium hydrogen phosphate 11	43.00 ± 5.6200	2.6 ± 1.0310
Ca^{2+}	730 ± 5.0125	19 ± 0.5012	Disodium hydrogen phosphate 12	45.00 ± 5.6200	2.2 ± 1.0310
Na^{+}	657 ± 5.0122	21 ± 0.5012	Control medium[k]	1461 ± 5.6092	38 ± 1.0863
Control medium[f]	812 ± 5.0122	19 ± 0.5014	Basal medium	306.0 ± 2.5611	2.4 ± 0.1283
Basal medium	305 ± 2.5250	2.3 ± 0.1250			
Temperature ºC					
20	269.0 ± 6.0117	30 ± 1.0700			
30	1512 ± 6.0372	45 ± 1.0700			
35	2472 ± 6.0298	50 ± 1.0701			
40	350.0 ± 6.0138	8.0 ± 1.0701			
50	149.0 ± 6.0472	5.0 ± 1.0700			
Control medium[g]	1512 ± 6.0372	45.4 ± 1.070			
Basal medium	319.0 ± 2.5611	2.4 ± 0.1830			

[a]mean ± SD. [b]basal medium without carbon source. [c]1% (w/v) sucrose and 0.5% (w/v) peptone without any inorganic nitrogen source.[d]1% (w/v) sucrose and 0.5% (w/v) peptone. [e]1% (w/v) sucrose and 0.5% (w/v) Meat extract without any inorganic nitrogen source. [f]1% (w/v) sucrose, 0.5% (w/v) Meat extract and 1.5% (w/v) without any metal ions. [g]The control medium was incubated at 30ºC. [h]1% (w/v) sucrose, 0.5% (w/v) Meat extract, 1.5% (w/v) and 2.5 mM (v/v) Zn^{2+} without any surfactants. [i]The medium was inoculated with the 6% (v/v) of the inoculums. [j]The medium was agitated at 150 rpm.[k]The pH of the control medium was adjusted to 6.5.