ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 8 (4): 425-433, 2009
ISSN 1682-296X

Optimization of Fermentation Conditions for the Biosynthesis of Inulinase by the New Source; *Aspergillus tamarii* and Hydrolysis of Some Inulin Containing Agro-Wastes

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Abstract: From the rotted Jerusalem artichoke tubers, 11 fungi were isolated on synthetic medium containing inulin as a sole carbon source. On the base of inulinase activity on inulin (I), one of them was selected and identified as *Aspergillus tamarii* AR-IN9. Incubation of *A. tamarii* AR-IN9 for 72 h, pretreatment of inulin-containing agro-wastes in autoclave at 20 Ib/in^2, 3% corn steep liquor in the growth medium, pH 5.5 and 35°C were the best conditions for inulinase production. The overall production reached up to 71.97 U mL^{-1}. *Aspergillus tamarii* AR-IN9 showed invertase activity on sucrose (S), with high values of I/S ratio which indicating that the fungus is active in inulinase production. Inulinase activity reached its maximum at pH 5.2 and 45°C. The enzyme was still stable by 80% or more at the pH range from 4.4 to 7.2 for 24 h and by 75% at 50°C for 90 min. The metal ions; $MgCl_2$, $CoCl_2$ and $MnCl_2$ positively modulated inulinase activity. The resultant inulinase showed high hydrolysis activity on Jerusalem artichoke (71.64%), dahlia tubers (67.55%) and chicory roots (55.11%). Therefore, various agro-wastes and inulin-containing materials could be economically hydrolyzed with *A. tamarii* AR-IN9 inulinase into fructose, which has many therapeutic and industrial aspects. Besides the beneficial environmental impact by the bioremediation of such agro-wastes.

Key words: *Aspergillus tamarii*, inulinase, invertase, agro-wastes, pretreatment

INTRODUCTION

Inulinases can be found in higher plants (Vandamme and Deryke, 1983; Kaur *et al*., 1992) and microorganisms as bacteria (Selvakumar and Pandey, 1999), yeasts (Singh *et al*., 2007) and fungi e.g., *A. niger* (Kumar *et al*., 2005) and *A. versicolor* (Kochhar *et al*., 1997). Inulinases belong to the group of fructanohydrolases, which, act as β-fructosidases and hydrolyze inulin to liberate fructose in a single step from the non-reducing end of fructosidic chains (Shady *et al*., 2000). Endoinulinase (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) is specific for inulin and hydrolyze the internal β-2,1 fructofuranosidic linkages to yield inulotriose, -tetraose and -pentaose as the main products. In contrast, exoinulinase (β-D-fructan fructohydrolase; E.C.3.2.1.80) splits terminal fructose units in sucrose, raffinose and inulin (Ohta *et al*., 2002).

Inulinases are used in the production of fructose syrup which have gained importance as the sweetest natural sugar and alternative to sucrose, especially for diabetes patients (Nakamura *et al*., 1995; Cruz *et al*., 1998). Inulinases have also been used for the production of inulo-oligosaccharides; the low caloric saccharides acting as growth factors for beneficial microorganisms in the intestinal flora (Skowronek and Fiedurek, 2006). Another application of inulinases is the production of ethanol from inulin (Ohta *et al*., 1993). Many microbial preparations of inulinase posses remarkable invertase activity, their catalytic activity is described in terms of inulin/sucrose ratio (Ettalibi and Baratti, 1987; Kango, 2008). Both types of enzymes are β-fructosidases which are active on sucrose but, in addition, inulinases are also able to hydrolyze inulin (Ettalibi and Baratti, 2001).

Inulin is a fructan, occurring as a reserve carbohydrate in the roots and tubers of plants like Jerusalem artichoke, chicory and dahlia, the residues of such plants could be used as a substrate for inulinase production (Jing *et al*., 2003). Production of fructose by acid hydrolysis of inulin is not recommended because of undesirable coloring of inulin hydrolysate, formation of difructose anhydride: which has practically no sweetening properties (Vandamme and Derycke, 1983; Kumar *et al*.,

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2005). Additionally, conventional fructose production from starch needs at least three enzymatic steps, which includes the action of α-amylase, amyloglucosidase and glucose isomerase and yields only 45% fructose (Mazutti *et al*., 2006). In contrast, specific inulinases rapidly and completely hydrolyze inulin in a single step giving a yield of about 90 to 95% fructose (Singh *et al*., 2007). Therefore, inulinase has received much attention for the production of fructose syrup from inulin (Nakamura *et al*., 1995; Shady *et al*., 2000).

Studying the factors controlling application of some plant residues and agro-wastes in inulinase production from native isolate of *Aspergillus tamarii* and the efficiency of such enzyme in hydrolyzing inulin-containing materials into fructose was the aim of this study.

MATERIALS AND METHODS

Agro-wastes and plant residues: Tubers of Jerusalem artichoke were obtained from Al-Mansoura Horticulture Research Station, Agric. Res. Center, Egypt. Tubers were peeled and cut into slices, vacuum dried at 30°C and packed in laminated polyethylene packs. The other agricultural wastes were cleaned, dried at 70°C and blended.

Isolation technique and screening for fungal inulinase: Samples of rotted Jerusalem artichoke tubers were used as a source for isolation of inulinase producing fungi, 10 g of rotted tubers was added to 100 mL sterilized distilled water, shaked for 30 min and serially diluted. The isolation technique was carried out on inulin agar plates (Kumar *et al*., 2005) using 1 mL from the diluted samples. On the base of ratio between clear zone diameter to colony diameter, 11 isolates were picked up and further assayed on the broth of the same medium to test inulinase activity. By observing the macroscopic and microscopic characteristics, the most potent inulinase producing fungus was identified as *A. tamarii* AR-IN9 (Domsch *et al*., 1980). The culture was maintained on PDA slants and subcultured weekly.

Inoculum preparation and fermentation technique: The fungus was subcultured on plates of PDA and incubated at 30°C for 72 h, resulted spores were suspended in sterile distilled water to approximately 2.5×10^6 spore mL^{-1} as determined by haemocytometer. Erlenmeyer flask containing 50 mL liquid medium of Ohta *et al*. (2002) which contains: inulin (BDH Chemicals Ltd, Pool, England), 1%; corn steep liquor (CSL), 2%; $(NH_4)H_2PO_4$, 1.2%; KCl, 0.07%; $MgSO_4.7H_2O$, 0.05% and $FeSO_4.7H_2O$, 0.001% and pH 4.8, was inoculated with 5% of the spore suspension. Incubation was carried in a rotary shaker at 200 rpm for 5 days at 30°C unless otherwise specified.

Pretreatment of inulin-containing agro-wastes: To get maximum yield of inulin from the inulin-containing agro-wastes; Artichoke tubers, dahlia tubers and chicory roots, they were processed by five pretreatment methods (Singh *et al*., 2007). In each method, 30 g of every agro-waste was added to 100 mL distilled water, the pretreatment lasted for 10 min, they are 1) vigorous shaking at room temperature in distilled water, 2) boiling in distilled water and 3, 4 and 5) extraction in distiller water under pressure (10, 20 and 30 Ib/in^2) in autoclaved. The pretreated inulin-containing agro-wastes were completed to a total volume of 1000 mL and used as a whole or after filtration (through ordinary filter paper) in the fermentation medium as a sole carbon source for inulinase production.

Optimization studies: The effects of the following parameters were investigated for the production of inulinase, they are: (a) incubation time (12 to 96 h), (b) sugars and agro-wastes as carbon and energy sources, (c) various concentrations of inulin-containing agro-wastes; Artichoke tubers, dahlia tubers and chicory roots, (d) pretreatment of inulin-containing agro-wastes, (e) nitrogen source, (f) concentration of CLS, (g) initial culture pH (4 to 8.0) and incubation temperature (20 to 45). After incubation period, the mycelium was filtered through Whatman No. 1 filter paper and dried at 105°C until constant weight to determine the biomass. The culture filtrate was centrifuged at 3000 rpm for 20 min and used as a source of extracellular inulinase and invertase.

Assays of inulinase and invertase activity: Inulinase activity on inulin (I) is commonly compared with the invertase activity on sucrose (S) displayed by the same enzyme preparation and the I/S ratio is used to characterize inulinases (Ohta *et al*., 2002). The inulinase and invertase activities were assayed by measuring reducing sugars released from inulin and sucrose, respectively. The reaction mixture contained 2 mL of 0.2% (w/v) inulin or 50 mM sucrose, 0.5 mL of the enzyme solution and 2.0 mL sodium acetate buffer (pH 4.8). Incubation was performed at 40°C for 20 min. After incubation, the tubes were kept in a boiling water bath for 10 min to inactivate enzyme. Reaction mixture was assayed for reducing sugars according to Somogyi (1952). One inulinase unit was defined as the amount of enzyme liberating one micromole of fructose equivalent per minute in the conditions used. The invertase unit was the amount of enzyme, which catalyzed the hydrolysis of one micromole of sucrose per minute. The amount of reducing

sugars was estimated by comparison with a calibration curves made with fructose for inulinase and equimolar solution of glucose and fructose for invertase (Ettalibi and Baratti, 1987).

Inulinase properties: Optimum pH and temperature of inulinase activity were investigated. For pH, the crude enzyme was incubated with inulin solutions prepared in 0.1 mol L^{-1} sodium acetate (pH 3.6 to 5.6), 0.1 mol L^{-1} phosphate (pH 6.0-7.8) or Tris-HCl (pH 8.2-9.0) buffers to cove a pH range from 3.6-9.0 at 40°C. For temperature, inulinase activity was determined after incubation at 25 to 65°C. The specific activity was defined as the rate between total enzyme activity and quantity of protein in milligram in one milliliter. Protein was determined according to Lowry *et al.* (1951), using crystalline bovine serum albumin as standard.

The pH and thermal stabilities of inulinase were determined. The enzyme solution was pre-incubated at various pH (3.6 to 9) at 30°C for 24 h and the residual activity was assayed under the standard conditions. Thermal stability after incubation at 20 to 80°C at different intervals (30, 60 and 90 min) in the absence of substrate was determined by measuring the residual activity of the enzyme.

The effect of different metal ions (at 1 mM) on inulinase activity was examined, incubating various metal salts with enzyme extract in sodium acetate buffer (pH 5.2) at 30°C for 1 h. The enzyme activities remaining after incubation were determined under assay conditions.

Hydrolysis of inulin-containing agro-wastes: Different inulinase units (10, 20 and 30 U) were added to 0.25 mL (4% w/v) of inulin, Jerusalem artichoke, dahlia tubers or chicory roots and 0.75 mL of sodium acetate buffer, pH 5.2 and incubated at the optimum temperature of the enzyme (45°C) for 15, 30, 60, 90 and 120 min. The degree of hydrolysis was expressed as the percent of reducing sugar against the total sugar×100. Total sugar in the hydrolyzed products was assayed according to Scott and Melvin (1953).

RESULTS AND DISCUSSION

Screening of inulinase producing fungi: A total of 11 fungi, isolated from the rotted Jerusalem artichoke tubers, solubilized inulin particles and exhibited clear surrounding zones on inulin agar plates. These isolates were assayed on liquid medium for 4 days. Of them, two isolates (AR-IN2 and AR-IN9) showed reasonable inulinase activity. Maximum activity (20.37 U mL^{-1}) was obtained by AR-IN9 isolate, which was identified as *A. tamarii* AR-IN9 according to Domsch *et al.* (1980) and used for detailed optimization studies.

Factors controlling enzyme biosynthesis

Time course of enzyme production: Initially, it was of interest to determine invertase activity together with inulinase, as many microbial preparations of inulinase posses remarkable invertase activity (S) accompanying the inulinase activity (I), their catalytic activity is described in terms of I/S (inulin/sucrose) ratio (Ettalibi and Baratti, 1987; Kango, 2008).

Results in Fig. 1 clearly show pronounced inulinase and invertase production with the increasing of fermentation period up to 72 h and then decreased. The maximum activities of inulinase (23.63 U mL^{-1}) and invertase (22.33 U mL^{-1}) were gained at the end of logarithmic phase of growth. There was a significant positive correlation (r) coefficient at $p \leq 0.01$ between growth from one side and the production of inulinase ($r = 0.954$) and invertase ($r = 0.963$) from the other side, in addition, inulinase and invertase were also positively correlated ($r = 0.994$). It has been reported that inulinase synthesis from *A. niveus* Blochwitz 4128URM is growth associated and reaches in the optima near the stationary phase (Souza-Motta *et al.*, 2005). The decline in enzyme activity after 72 h of fermentation could be either due to decrease in nutrient availability in the medium or catabolic repression of enzyme. These results are similar to those obtained by Kumar *et al.* (2005). On the other hand, Kochhar *et al.* (1997) reported maximum yield of inulinase with *A. versicolor* after 15 days of growth.

Related to pH, the final culture pH of the media showed high acidification (Fig. 1), the pH decreased to approximately 3.5 after 72 h. Souza-Motta *et al.* (2005) reported that during the inulinase production by *A. niveus*, the pH values decreased from 5 to 2.5 after 96 h due to formation of organic acids.

Carbon source: Various carbon sources, including inulin-containing agro-wastes, were added to the production medium instead of inulin to study their effect on inulinase production by *A. tamarii* AR-IN9 (Table 1). Inulinase was

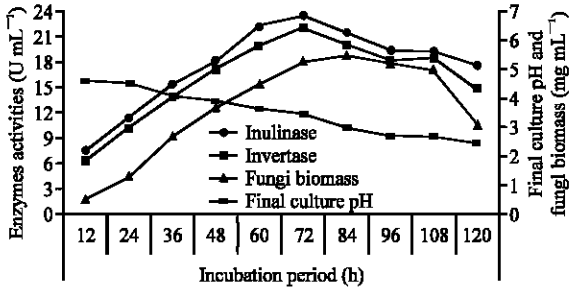

Fig. 1: Inulinase and invertase activities, final pH and growth curve of *A. tamarii* AR-IN9 as a function of time

Table 1: Production of *A. tamarii* AR-IN9 inulinase and invertase on different carbon sources

Carbon source (1%)	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio
Glucose	10.24	9.22	1.11
Fructose	15.71	18.10	0.87
Maltose	7.66	5.05	1.52
Sucrose	14.00	18.50	0.76
Raffinose	19.33	8.00	2.42
Starch	13.87	11.23	1.24
Inulin	23.63	11.33	2.09
Artichoke tuber	23.30	19.10	1.22
Dahlia tuber	26.50	19.90	1.33
Chicory root	25.80	12.57	2.05
Beet pulp	19.00	11.21	1.69
Orange peel	20.83	12.23	1.70
Cane molasses	13.17	13.01	1.01
Beet molasses	15.87	14.24	1.11

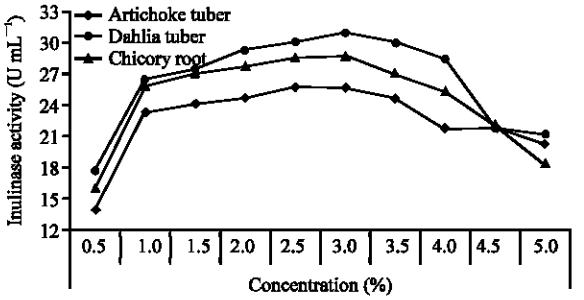

Fig. 2: Inulinase production by *A. tamarii* AR-IN9 on different concentrations of inulin-containing agro-wastes

produced on the tested carbon sources, but with different extents. Among various substrates employed, inulin-containing agro-wastes offer advantages in comparison to pure substrates, in this respect, dahlia tuber, chicory roots and artichoke tuber enhanced inulinase production in descending order, in which the enzyme activities reached 26.5, 25.8 and 23.3 U mL^{-1}, respectively. Fructose, which is believed to be the primary inducer of inulinase (Jing *et al*., 2003), produced an inulinase yield that was about 66% of that observed with inulin (Table 1). This is an indication that inulin is a potential inducer of inulinase. Other carbon sources such as glucose, maltose and starch decreased inulinase production. Hence, this enzyme has constitutive nature and could be also, induced by its substrate. This was evidenced from the use of all other carbon sources, which stimulated inulinase production beyond basal level. These results are in agreement with those obtained by Fontana *et al.* (1994) and Shady *et al.* (2000). On the other hand, Vandamme and Derycke (1983) reported that microbial inulinases are usually inducible.

The results also, show activity of invertase especially on sucrose, the ideal substrate of invertase (I/S ratio reduced to 0.76). The I/S ratio on inulin-containing agro-wastes ranged from 1.22 to 2.05 compared with 2.09 on inulin. Kango (2008) noticed that I/S ratio varied significantly (0.12 to 6.6) among all the carbon sources examined along with inulinase levels. It is generally accepted that the ratio of the activity on inulin/sucrose (I/S ratio) characterizes the enzymes: for inulinase I/S ratio is higher than 10^{-2} while for invertase it is lower than 10^{-4} (Guiraud, 1981). More detailed studies described three different types of β-fructofuranosidases (invertase, exoinulinases and endoinulinases) from *A. ficuum* based on their molecular weight, mode of action on inulin and I/S ratios. The invertase had a molecular weight of 84000 and was much more active on sucrose than on inulin: the I/S ratio was 0.01. The exoinulinase liberated free fructose and had a molecular weight of 74000 and I/S ratio in the range 0.16-0.36. The endoinulinase gave oligofructosides and had a molecular weight of 64000 with I/S ratio in the range 0.86 to 2.92 (Ettalibi and Baratti, 1987). The main characteristic of endoinulinase was the lack of detectable hydrolysis of sucrose, this is a unique property of all the described microbial inulinases (Vandamme and Derycke, 1983). According to these classifications, the present investigation characterizes inulinase activity since I/S ratio ranged from 0.76 to 2.42 (Table 1).

Since, inulin-containing agro-wastes were the most inducers of inulinase production, their concentrations were screened. The results of Fig. 2 show progressive increase in enzyme production with the increase of concentration up to 2.5% for artichoke tuber (25.71 U) and 3% for both dahlia tuber (31.0 U) and chicory roots (28.61 U), thereafter, declined. It has been well established that higher substrate concentration can lead to catabolic repression, consequently lowering the inulinase activity (Jing *et al*., 2003). The results are in agreement with those of Shady *et al.* (2000) and Singh *et al.* (2007).

Inulinase production on the pretreated inulin-containing agro-wastes: Inulin-containing agro-wastes were further investigated by studying the relation between the methods of pretreatment and inulinase production by *A. tamarii* AR-IN9 (Table 2). Maximum inulinase production (36.17 U mL^{-1}) was obtained by growing *A. tamarii* AR-IN9 on medium containing dahlia that was pretreated by presser at 20 Ib/in^2 for 10 min without filtration, followed by chicory roots (31.79 U mL^{-1}) then artichoke tuber (28.55 U mL^{-1}). However, the production of both inulinase and invertase on a medium prepared from the filtrates of pretreated inulin-containing agro-wastes were slightly lower as compared to using the whole pretreated agro-wastes without filtration in the fermentation medium. This could be attributed to the presence of inulin-containing residues, which may acts as reservoir for inulin that released slowly during the

Table 2: Effect of pretreatment method of inulin-containing agro-wastes on enzymes production

Pretreatment method (30 g agro-waste in 100 mL water for 10 min)	Filtrate of pretreated plant*			Whole pretreated plant**		
	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio
Artichoke tuber						
Shaking	10.71	2.10	5.10	25.71	20.10	1.28
Boiling	15.97	4.31	3.71	25.94	21.33	1.22
In autoclave (Ib/in^2)						
10	19.13	9.67	1.98	27.33	23.94	1.14
20	20.24	8.99	2.25	28.55	24.79	1.15
30	18.44	7.22	2.55	26.91	24.12	1.12
Dahlia tuber						
Shaking	17.17	8.91	1.93	31.70	21.71	1.46
Boiling	25.60	9.10	2.81	32.43	23.01	1.41
In autoclave (Ib/in^2)						
10	27.43	15.88	1.73	33.73	25.05	1.35
20	29.00	17.56	1.65	36.17	26.43	1.37
30	26.47	17.15	1.54	32.77	26.01	1.26
Chicory root						
Shaking	13.12	10.00	1.31	28.77	15.34	1.88
Boiling	16.43	12.26	1.34	29.21	16.30	1.79
In autoclave (Ib/in^2)						
10	22.57	13.15	1.72	30.31	21.11	1.44
20	25.48	13.32	1.91	31.79	23.12	1.38
30	21.78	11.23	1.94	30.97	21.03	1.47

*Using extraction of the pretreated plant in the fermentation media after filtration. **Using the whole pretreated plants in the fermentation medium without filtration

Table 3: *A. tamarii* AR-IN9 inulinase and invertase production as influenced by nitrogen source in combined with agro-wastes

Nitrogen source	Artichoke tuber			Dahlia tuber			Chicory roots		
	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio
Ammonium phosphate	21.15	11.65	1.82	24.27	13.31	1.82	23.31	10.10	2.31
Sodium nitrate	15.56	10.22	1.52	13.30	12.88	1.03	15.56	12.21	1.27
Potassium nitrate	10.98	5.120	2.14	14.23	13.77	1.03	16.99	4.450	3.82
Ammonium chloride	13.61	6.610	2.06	14.10	10.85	1.30	15.60	4.550	3.43
Casein	16.66	3.140	5.31	18.50	5.540	3.34	17.30	3.500	4.94
Gelatin	16.85	4.540	3.71	16.83	5.980	2.81	10.22	5.120	2.00
Yeast extract	28.40	25.46	1.12	35.51	32.14	1.10	27.46	25.40	1.08
Tryptone	15.37	2.150	7.15	16.53	6.550	2.52	16.55	6.670	2.48
Peptone	19.91	15.60	1.28	21.53	16.67	1.29	20.48	18.87	1.09
CSL	28.51	27.43	1.04	36.33	33.89	1.07	32.09	25.64	1.25
Control	28.50	24.79	1.15	36.17	26.43	1.37	31.79	23.12	1.38

fermentation process. These results are in agree with those of Singh *et al*. (2007) who found that processing of dahlia at 15 Ib/in^2 led to increase inulin content in the extraction; this raw inulin could be used to economize inulinase production. In addition, Kango (2008) reported that extracts of Dandelion root, garlic and onion contain inulin and oligofructans and possibly this fructan component induced maximal inulinase production as compared to pure inulin and other complex substrates. The results also, declared that I/S ratio was not constant and varied even within the agro-waste. This could be explained by the existence of two separate enzymes for inulin and sucrose hydrolysis with different sensitivities to the growth conditions (Allais *et al*., 1986).

Nitrogen source: Nitrogen sources were added to the production medium instead of the initial nitrogen source (CSL+ (NH_4) H_2PO_4) with the same equivalent nitrogen content. Results of Table 3 reveal that whatever the source of nitrogen used, inulinase production was supported on the tested inulin-containing agro-wastes, but with different extents. Of organic N-sources, CSL and yeast extract, maximally, stimulated inulinase biosynthesis by *A. tamarii* AR-IN9. The inorganic N-sources seemed to be not favorable for inulinase production. Kumar *et al*. (2005) reported CSL as the best organic nitrogen source for maximum production of *A. niger* AUP19 inulinase. Invertase showed positive correlation with inulinase production at $p \leq 0.01$ on the tested inulin-containing agro-wastes; artichoke tuber ($r = 0.928$), dahlia tuber ($r = 0.890$) and chicory root ($r = 0.865$). Moreover, values of I/S ratio lied within a range that characterizing inulinase activity (I/S ratio$>10^{-2}$) according the accepted classification of Guiraud (1981) and Ettalibi and Baratti (1987). Variation in I/S ratio, ranging from 4.7 to 9.5, with respect to nitrogen source, has been observed with *Penicillium* sp. TN-88 by Nakamura *et al*. (1997).

Since, there is no marked differences between the control (CSL+ (NH_4) H_2PO_4) and CLS alone in inulinase

production, the later was tested at various concentrations (0.5 to 5%) as a sole nitrogen source in combined with different inulin-containing agro-wastes. Figure 3 shows that inulinase productivity increased gradually with the increasing of CSL up to 3 % by dahlia tuber (41.97 U mL^{-1}) and chicory root (35.99 U mL^{-1}) and 2.5% by artichoke tuber (33.41 μ mL^{-1}), then decreased. This could be due to the complex nature of CLS and some of its constituents at higher concentration might have a toxic effect on inulinase production. Among the tested inulin-containing agro-wastes, dahlia tuber was the best in inulinase production. The increment in inulinase activity with CSL concentrations may be due to the increase in fungal growth (Viswanathan and Kulkarni, 1995). The results are in accordance with those of Shady *et al*. (2000).

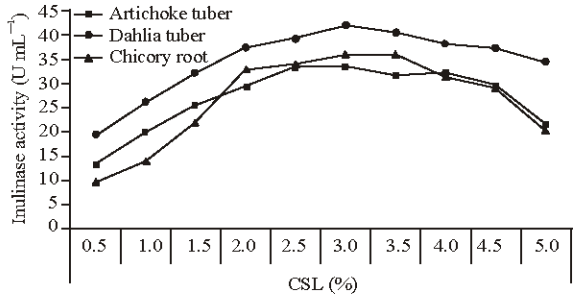

Fig. 3: Corn steep liquor concentration against *A. tamarii* inulinase production on different inulin-containing agro-wastes

Initial culture pH and incubation temperature: Of the various pHs and temperatures tested (Table 4, 5), maximum inulinase production was attained at pH 5.5 and 35°C, indicating that the isolated *A. tamari* is acidophilic and mesosphilic fungus, respectively. Moreover, *A. tamarii* AR-IN9 showed significant inulinase production in the range of pH 4.5 to 7.0 and temperature of 30 to 40°C. Out of these conditions, the inulinase production decreased. Similar results were obtained by Shady *et al*. (2000). Kumar *et al*. (2005) found that pH 6.5 and 28°C were the optimum for *A. niger* AUP19 inulinase production. With respect to invertase production, pH 5.0 and 30°C were found to be optimum for its maximum production. It is of special interest to note that there was remarkable inconstancy in I/S ratio. Allais *et al*. (1986) explained the inconstancy of I/S ratio on the base of the presence of inulin and sucrose hydrolysis enzymes with different sensitivities to pH.

Inulinase properties: During the optimization studies, dahlia tubers was found to be the most inducer for both inulinase and invertase production. So, enzyme preparation obtained from *A. tamarii* AR-IN9 grown on dahlia tubers was used for studying inulinase properties.

Inulinase activity as influenced by pH and temperature: Inulinase activity was studied at different pH and temperature degrees. The results (Table 6, 7) show that inulinase could hydrolyze inulin at wide ranges of pH (4.4 to 6.4) and temperature (40 to 55°C), with optimum at pH 5.2 and 45°C, respectively. Out of these ranges, the

Table 4: Effect of initial culture pH on *A. tamarii* AR-IN9 inulinase production on different agro-wastes

	Artichoke tuber			Dahlia tuber			Chicory roots		
Initial culture pH	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio
4.0	20.33	20.20	1.01	27.33	10.35	2.64	20.11	18.00	1.12
4.5	33.00	28.12	1.17	41.40	26.79	1.55	35.04	21.40	1.64
5.0	37.88	30.18	1.26	43.47	33.71	1.29	37.76	30.05	1.26
5.5	39.41	27.62	1.43	49.23	31.71	1.55	38.99	29.94	1.30
6.0	39.03	25.13	1.55	47.73	30.30	1.58	33.13	23.28	1.42
6.5	36.11	20.76	1.74	46.53	29.46	1.58	23.18	19.07	1.22
7.0	31.50	15.13	2.08	38.53	22.12	1.74	19.28	15.15	1.27
7.5	21.14	14.33	1.48	25.53	10.00	2.55	11.17	9.92	1.13
8.0	10.01	14.00	0.72	13.73	5.50	2.50	8.12	5.13	1.58

Table 5: Effect of incubation temperature on *A. tamarii* AR-IN9 inulinase production on different agro-wastes

	Artichoke tuber			Dahlia tuber			Chicory roots		
Temperature (°C)	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio	Inulinase (U mL^{-1})	Invertase (U mL^{-1})	I/S ratio
20	14.70	10.89	1.35	16.47	12.90	1.28	15.33	11.24	1.36
25	24.92	20.52	1.21	30.17	23.70	1.27	20.55	21.74	0.95
30	39.41	27.62	1.43	49.23	33.15	1.49	38.99	29.94	1.30
35	42.85	27.00	1.59	57.07	33.00	1.73	40.74	28.80	1.41
40	31.64	25.59	1.24	42.73	30.70	1.39	29.90	20.44	1.46
45	21.81	23.89	0.91	30.80	21.70	1.42	17.32	15.65	1.11

Table 6: Effect pH on *A. tamarii* AR-IN9 inulinase activity

pH	Inulinase activity (U mL^{-1})*	Specific activity (U mg $protein^{-1}$ mL^{-1})**	Relative activity (%)
3.6	11.03	18.79	19.33
4.0	32.70	55.71	57.30
4.4	53.01	90.31	92.89
4.8	57.07	97.22	100.00
5.2	68.41	116.54	119.87
5.6	61.61	104.96	107.96
6.0	59.37	101.14	104.03
6.4	55.15	93.95	96.64
6.8	43.33	73.82	75.92
7.2	30.26	51.55	53.02
7.4	12.35	21.04	21.64
7.8	10.10	17.21	17.70
8.2	7.14	12.16	12.51
8.6	5.01	8.53	8.78
9.0	3.22	5.49	5.64

*The temperature of the reaction mixture is 40°C. **Protein content of the culture fluid is 0.587 mg mL^{-1}

Table 7: Effect of temperature on *A. tamarii* AR-IN9 inulinase activity

Temperature (°C)	Inulinase activity (U mL^{-1})*	Specific activity (U mg $protein^{-1}$ mL^{-1})**	Relative activity (%)
25	15.47	26.35	22.61
30	40.33	68.71	58.95
35	51.50	87.73	75.28
40	68.41	116.54	100.00
45	71.97	119.91	102.89
50	70.99	122.61	105.20
55	70.39	120.94	103.77
60	53.81	91.67	78.66
65	48.01	81.79	70.18

*The pH of the reaction mixture is 5.2. **Protein content of the culture fluid is 0.587 mg mL^{-1}

specific activity decreased. These ranges of pH and temperature of inulinase activity may make this enzyme suitable for industrial processes that depending on pH and/or temperature. Ongen-Baysal *et al.* (1994) recorded maximum inulinase activity at pH between 5.0 to 6.0 at 50°C and Cruz *et al.* (1998) reported pH 4.0 to 4.5 at 60°C for maximum inulinase activity by *A. niger*. However, temperatures above 55°C could inactivate some inulinases produced by fungi (Vandamme and Derycke, 1983; Souza-Motta *et al*., 2005).

Higher temperature optimum of inulinases is an extremely important factor for the application of these enzymes for commercial production of fructose or fructooligosaccharides from inulin, since high temperatures (60°C or higher) ensure proper solubility of inulin and prevent microbial contamination (Vandamme and Derycke, 1983; Singh and Gill, 2006).

For industrial application in obtaining concentrated fructose syrups, enzymes with larger activity in pH around to 5.0, as the one here described, are suitable since they make difficult the bacterial contamination of the process (Cruz *et al*., 1998).

pH and thermo stabilities of inulinase: The stability of inulinase against pH was studied exposuring inulinase to pH range of 3.6 to 9 (Fig. 4). The enzyme remained 80% or more of its activity at the pH range of 4.4 to 7.2, the stability of inulinase decreased out of this range. In accordance with Cruz *et al.* (1998), the inulinase from *A. niger* 245 showed whole stability in the pH range from 3.5 to 7.0.

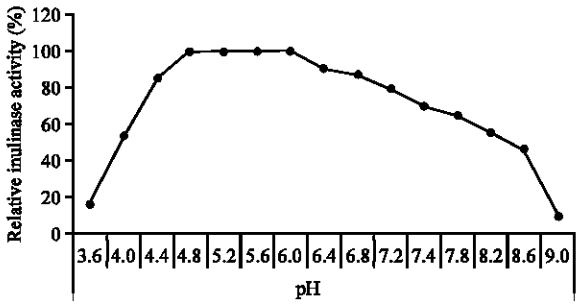

Fig. 4: pH stability of *A. tamarii* AR-IN9 inulinase

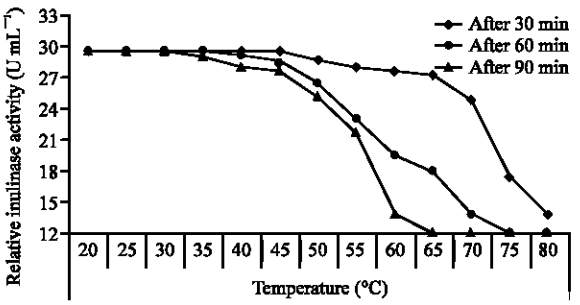

Fig. 5: Thermal stability of *A. tamarii* AR-IN9 inulinase

The thermoinactivation of inulinase at the temperature range of 20 to 80°C (Fig. 5) showed remarkable thermostability, which decreased by the time of exposure. The enzyme kept 100%, of its activity after 30 min of incubation at 45°C, the activity reduced to 94 and 89% by increasing time of exposure to 60 and 90 min, respectively. Higher thermostability of the industrially important enzymes also brings down the cost of production because lower amount of enzyme is required to produce the desired product (Vandamme and Derycke, 1983; Cruz *et al*., 1998). The inulin hydrolytic activity from *Aspergillus* sp. the most versatile sources of inulinases; is more thermostable (Singh and Gill, 2006).

Effect of metal ions on inulinase activity: The effect of various metal ions on inulinase activity was investigated (Fig. 6). Only $MgCl_2$, $CoCl_2$ and $MnCl_2$ positively modulated inulinase activity, whereas the inhibitory effect of KCl, NH_4Cl_2 NaCl and $CaCl_2$ was marginal. The $HgCl_2$, which is known to affect thiol groups and $FeCl_2$ completely, abolished the inulinase activity. The strong inhibitory effect observed with Hg^{2+} suggested that some -SH-group in the protein might be essential for the

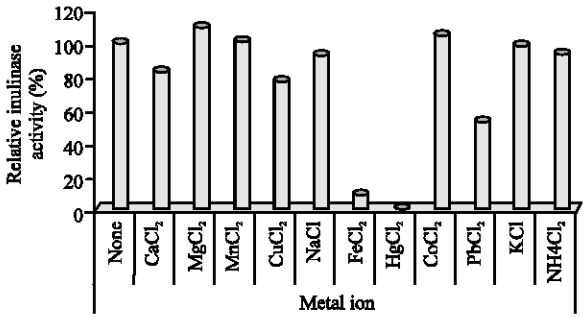

Fig. 6: Effect of metal ions on the activity of *A. tamarii* AR-IN9 inulinase

Table 8: Hydrolysis of some inulin-containing agro-wastes by *A. tamarii* AR-IN9 inulinase

		Degree of hydrolysis (%)			
Inulinase units	Time of hydrolysis (min)	Inulin	Artichoke tuber	Dahlia tuber	Chicory root
10	15	6.97	9.08	8.14	6.55
	30	13.43	14.57	19.90	13.12
	60	19.17	20.10	22.05	17.75
	90	25.80	27.88	28.15	22.01
	120	31.22	36.10	37.80	29.16
20	15	15.10	17.90	17.17	13.73
	30	17.33	19.21	19.50	15.31
	60	31.50	38.90	31.67	26.67
	90	42.80	47.50	40.33	30.33
	120	45.33	51.37	43.67	38.44
30	15	23.99	25.33	23.17	20.15
	30	38.69	37.16	33.65	27.15
	60	44.90	53.66	40.41	33.67
	90	53.50	59.50	51.33	49.67
	120	66.81	71.64	67.55	55.11

activity. This has been earlier observed for other microbial inulinases also (Ettalibi and Baratti, 1987; Kochhar *et al*., 1997; Sharma *et al*., 2006). Ohta *et al*. (2002) reported stimulation of the inulinase activity by Mn^{2+} and Ca^{2+} and completely inactivation by Hg^{2+}. On the other hand, Mg^{2+} and Zn^{2+} did not influence the enzyme activity.

Hydrolysis of some inulin containing agro-wastes by *A. tamarii* AR-IN9 inulinase: The hydrolysis of inulin-containing materials was carried out at the optimum temperature (45°C) and pH (5.2) of the enzyme, using 10, 20 and 30 units of inulinase at different time intervals (Table 8). Inulinase could hydrolyze various substrates but the degree of hydrolysis (DH) varied. In general, there are directional relationships between DH, from one side and inulinase units and time of hydrolysis, from the other side. *A. tamarii* AR-IN9 inulinase showed preference in hydrolysis Jerusalem artichoke (71.64%) and dahlia tubers (67.55%) than chicory roots (55.11%) compared to inulin (66.81%) after 120 min with 30 units of inulinase.

During the hydrolysis of such agro-wastes by *A. tamarii* AR-IN9 inulinase, reducing sugars produced, this is due to the action of inulinase in production of fructose in a single step from the non-reducing end of fructosidic chains (Shady *et al*., 2000). The variation of hydrolyzing ability of inulinase on some inulin-containing agro-wastes and plant materials may be due to the difference in affinity between each substrate and enzyme. This may also, be back to the degree of polymerization and the higher polyfructans in pure inulin as well as the presence of other ingredient in the inulin-containing materials (Nakamura *et al*., 1995).

So, *A. tamarii* AR-IN9 inulinase may be used for economically converting agro-wastes and plant residues that contain inulin such as dahlia tubers and Jerusalem artichoke into fructose, especially Jerusalem artichoke that rapidly spoiled after harvesting. Besides the beneficial environmental impact by the bioremediation of such agro-wastes.

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