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Aspergillus kawachii produces an inulinase in cultures with yacon (*Smallanthus sonchifolius*) as substrate

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Abstract

Background: Inulinases have been extracted and characterized from inulin-storing tissues; however, production of microbial inulinases have recently draw much attention as they offer several industrial advantages. Many microorganisms, including filamentous fungi, yeast and bacteria have been claimed as inulinase producers. These hydrolases are usually inducible and their exo-acting forms may hydrolyze fructose polymers (inulin) and oligosaccharides such as sucrose and raffinose. Fungal inulinase extracts are often produced as stable mixture of highly active fructanhydrolases. From a practical prospective, the best known inulinases to date are those produced by species of Penicillium, Aspergillus and Kluyveromyces.
Results: The production of extracellular inulinase by *A. kawachii* in liquid cultures, using either inulin or yacon derived materials as CES as well as inulinase inducers, is reported. In addition, a partial characterization of the enzyme activity is included.
Conclusions: Yacon derived products, particularly yacon juice, added to the culture medium proved to be a good CES for fungal growth as well as an inducer of enzyme synthesis. Partial characterization of the enzyme revealed that it is quite stable in a wide range of pH and temperature. In addition, characterization of the reaction products revealed that this enzyme corresponds to an exo-type. These facts are promising considering its potential application in inulin hydrolysis for the production of high fructose syrups.

Keywords: acidophilic enzymes, batch cultures, inulin degradation, submerged cultures.

INTRODUCTION

Inulin consists of linear chains of β-2,1-linked D-fructofuranose residues attached to a terminal sucrose molecule (Vandamme and Derycle, 1983). Inulin is present as a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia and yacon. Such inulin sources have recently received a great deal of attention as they represent a renewable, low-cost and abundant raw material for the production of ultra-high fructose syrup. In addition, they can be used for bioethanol production as well as a versatile source for inulo-oligosaccharides, single-cell oils and proteins and some chemicals like citric acid, butanediol, alcohols and lactic acid (Pandey et al. 1999; Chi et al. 2009a; Liu et al. 2010; Chi et al. 2011).

Fructose obtained from inulin is produced in a single enzymatic step with yields as high as 95% (Vandamme and Derycle, 1983; Pandey et al. 1999). Inulin can be hydrolyzed by two different types of inulinases: exoinulinase (β-D-fructan fructohydrolase, EC 3.2.1.80) that liberates fructose; and endoinulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) that produces oligosaccharides (Shuichi and Norio, 1992; Skowronek and Fiedurek, 2004).

Inulinases have been extracted and characterized from inulin-storing tissues (Pandey et al. 1999); however, production of microbial inulinases have recently draw much attention as they offer several industrial advantages. Many microorganisms, including filamentous fungi, yeast and bacteria have been claimed as inulinase producers (Chi et al. 2009b). These hydrolases are usually inducible and their exo-acting forms may hydrolyze fructose polymers (inulin) and oligosaccharides such as sucrose and raffinose. Fungal inulinase extracts are often produced as stable mixture of highly active fructanhydrolases. From a practical prospective, the best known inulinases to date are those produced by species of *Penicillium* (Shuichi and Norio, 1992), *Aspergillu*s and *Kluyveromyces* (Pandey et al. 1999).

Aspergillus kawachii IFO 4308 is a fungus traditionally used in the Japanese food industry. It produces several acidic depolymerases, such as amylases and glucoamylases, xylanases, acidophilic proteinases and polygalacturonases (Vita et al. 2009). These enzymes show potential biotechnological applications because they are active and stable at low pH, which are advantageous characteristics for some industrial bioprocesses.

Yacon (*Smallanthus sonchifolius* Poepp. & Endl) is a perennial herb original from South America. It is cultivated as a root crop in the Andes from Colombia to north western Argentina at altitudes between 1000 and 3500 m. Its tuber roots contain only 0.3-3.7% protein, but 70-80% of its dry matter is composed of saccharides, manly fructooligosaccharides (FOS) (Campos et al. 2012). The underground storage organs of yacon accumulate over 60% (on dry basis) of inulin type β(2-1) fructans, mainly oligomers (GF_2-GF_{16}) (Itaya et al. 2002). Yacon roots have a long history of use in South America and other regions, having potential health promoting properties, including prebiotic, antidiabetic, antioxidative and antimicrobial effects (Ojansivu et al. 2011).

The presence of inulin in yacon tubercles motivated the interest for studying this plant material as a potential substrate for the growth of inulinase producing microorganisms. Previous screening experiments using a liquid minimal medium with different carbohydrates as sole carbon and energy sources (CES) demonstrated that *A. kawachii* is able to grow on inulin; therefore, the production of a certain type of inulinase by *A. kawachii* was postulated. In the present study, the production of extracellular inulinase by *A. kawachii* in liquid cultures, using either inulin or yacon derived materials as CES as well as inulinase inducers, is reported. In addition, a partial characterization of the enzyme activity is included.

MATERIALS AND METHODS

Materials. Glucose, sucrose and inulin were from Sigma Chemical Co. (St. Louis, MO, USA). Food-grade inulin (a kind gift from Droguería Saporiti, Argentina) was also used in some cases. All other chemicals used were commercial products of analytical grade.

Processing of yacon. Yacon samples were kindly provided by Prof. A. Grau, Universidad Nacional de Tucumán, Argentina. About 1 kg of tubercles were washed, peeled and squeezed using a lab-scale hydraulic press (maximum pressure: 300 kg cm^{-2}) until no more juice was released. The juice obtained was clarified by filtration (Whatman # 41) and kept frozen at -20ºC until used. Solids remaining in the hydraulic press after pressing (so called yacon pomace) were cut in squared pieces of ~0.25 cm^2, sterilized by UV radiation during 20 min and kept under aseptic conditions until used as CES (yacon pomace chips).

Microorganism and inoculum preparation. *Aspergillus kawachii* IFO 4308 spores were propagated, harvested and stored in a cryoprotector system at -80ºC. When needed, the strain was activated in Potato Dextrose Agar (PDA; Britania, Argentina) and incubated at 30ºC for seven days; spores were suspended in 0.01% Tween 80 solution and counted in a Neubauer chamber.

Culture conditions. Culture medium composition (in g/l) was: $NaNO_3$ (2.0), K_2HPO_4 (1.0), $MgSO_4$ x $7H_2O$ (0.5), KCl (0.5) and $FeSO_4$ x $7H_2O$ (0.01), pH: 5.0. Different carbon and energy sources (CES) were added (in g/l): glucose, sucrose or food grade inulin (10), yacon pomace chips (25) or yacon juice (25 ml/l). All medium components were autoclaved during 15 min with the exception of yacon juice which was sterilized by filtration through a cellulose filter paper (0.22 µm, E02WP02500, MSI, USA). In some cultures, initial pH of the salt solution was conveniently adjusted in order to obtain values from ~3.0 to ~6.0 after yacon juice addition.

Erlenmeyer cultures. Erlenmeyer flasks (1000 ml) containing 100 ml of medium were inoculated with 1.0×10^6 spores x ml^{-1} and incubated at 30ºC in a rotary shaker (250 rpm) for up to 7 days. Samples of 20 ml were taken at different intervals depending on the CES used and the mycelium was separated by filtration (Whatman # 41). Enzymatic crude filtrates were analyzed in terms of extracellular inulinase activity, pH, total sugars and fungal biomass depending on the purpose of the experiment.

Bioreactor cultures. Batch cultures were carried out in a 5-l fermentor (LH 210, Inceltech, France), using yacon juice as CES, according to the protocol above described for Erlenmeyer cultures, at 30ºC, with aeration (1.0 vvm) and agitation (450 rpm). Culture pH was measured with a glass electrode (Mettler-Toledo). Dissolved oxygen was measured with a polarographic-type electrode (Mettler-Toledo).

Fungal biomass determination. In cultures with yacon juice as CES, mycelial mass was recovered by filtration (Whatman # 41), washed with distilled water and dried at 105ºC until constant weight.

Total and reducing sugars content. Total and reducing sugars contents were determined according to the traditional colorimetric methods of phenol-sulphuric acid or Somogyi-Nelson, respectively, using fructose or glucose as standard.

Inulinase assay. Inulinase activity was assayed by measuring the amount of reducing sugars released from inulin as recommended by Gill et al (2006). For the assay, an appropriate dilution of enzyme sample (20 µl) was mixed with 180 µl of 0.5 g x l^{-1}inulin solution in 50 mM citric acid/25 mM Na_2HPO_4 buffer (CPB), pH 5, and incubated at 37ºC for 2 hrs. The reducing sugars released during the reaction were assayed following the Somogyi-Nelson methodology using fructose as standard. One unit of enzymatic activity (U) was defined as the amount of enzyme required to liberate 1 µmol of reducing sugars per minute under the assay conditions.

Partial characterization of inulinase activity

Effect of temperature and pH on inulinase stability and activity. Thermal stability of *A. kawachii* inulinase was measured in terms of residual activity after incubation of a culture filtrate sample in CPB (pH 5) at different temperatures (37ºC, 45ºC, 55ºC and 65ºC) for 180 min.

The stability of the enzyme at different pH values was determined by measuring the residual activity after incubation of a culture filtrate sample at 37ºC for 180 min in a mixture of buffers (Tris-HCl, MES, and Glycine, 20 mM each), adjusted to different pH values from 3 to 13.

The effect of pH on inulinase activity was determined by incubating 20 µL of suitably diluted culture filtrate sample in 180 µL of inulin solution (0.5 g x l^{-1}) in a mixture of buffers (Tris-HCl, MES, and Glycine, 20 mM each) adjusted to different pH values from 3 to 13.

Effect of metal ions on inulinase stability. The effect of different metal ions (K^+, Ca^{2+}, Hg^{2+}, Mn^{+2}, Zn^{2+} and Fe^{+3}) on inulinase activity was studied at a concentration of 1 mM by pre-incubating a culture filtrate sample with metal salts solutions for 1 hr at room temperature. The remaining inulinase activity was determined and expressed as a percentage of residual activity relative to a control (100%) without treatment.

Hydrolysis of inulin. A reaction mixture containing 1.8 ml (food grade) inulin solution (0.5 g x l^{-1}, in CPB, pH: 3.5) and 200 µL of crude culture filtrate (80 mU x ml^{-1}) was incubated at 37°C for 6 hrs. Sampling was carried out every 1 hrs. The products of inulin hydrolysis were determined qualitatively

by thin layer chromatography (TLC) using 8 cm-plates (Merck, TLC aluminum sheets 20 x 20 cm, Silica gel F_{254}). Samples (5 µl) of hydrolyzate were spotted on the pre-coated TLC plates. A mixture of ethyl acetate:acetic acid:water (3:1:1, v/v/v) was used as mobile phase. Plates were air-dried and carbohydrates were stained with 50:50 (w/w) sulphuric acid: methanol reagent. The plates were sprayed with the staining reagent and colour developed by heating for 5 min at 105ºC. By this procedure, glucose, fructose and sucrose were visualized as dark brown spots.

RESULTS AND DISCUSSION

Cultures of *A. kawachii*

Effects of different CES on inulinase production. Different carbohydrates, including glucose, fructose, sucrose, food grade inulin and yacon pomace chips and juice (prepared as described in materials and methods) were tested as CES for inulinase production in Erlenmeyer flaks cultures. A preliminary characterization of yacon juice revealed that total and reducing sugars contents were 80 g x l^{-1} and 70 g x l^{-1} respectively. *A. kawachii* grew well in all CES tested, in the form of small pellets of 1-2 mm in diameter. Nevertheless, values of inulinase activity achieved were quite different. As shown in Figure 1, inulin and yacon juice were the CES yielding the highest inulinase activities. On the contrary, inulinase activity was severely reduced by simple sugars suggesting the presence of catabolic repression over inulinase synthesis. The use of yacon pomace chips resulted in intermediate enzyme activity values, probably due to the low CES remaining levels in this material.

From these results, it can be tentatively postulated that inulinase from *A. kawachii* is an inducible enzyme, and that inulin (or closely related fructans present in yacon juice) act as specific inducers. A similar behaviour was found when studying the production of extracellular exoinulinase from *Clostridium acetobutylicum* that was significantly inhibited in the presence of glucose, fructose and sucrose, but induced in the presence of inulin (Singh et al. 2007b; Singh and Bhermi, 2008). Although inulin was proved to be the best carbon source for inulinase production from *Kluyveromyces marxianus* (Parekh and Margaritis, 1985), no repression of the synthesis of inulinase was observed in this yeast with other carbon sources. Furthermore, it was reported that the inulinase from *Xanthomonas campestris* was constitutively expressed and not inducible (Ayyachamy et al. 2007). Moreover, inulin has also been observed as a poor inulinase inducer in *Streptomyces sp.* GNDU (Gill et al. 2003) and *Aspergillus niger* 245 (Cruz et al. 1998). *Bacillus polymyxa* 722 and *Bacillus polymyxa* 29 displayed the maximum inulinase activity on a starch-containing culture medium, while the maximum activity of *Bacillus subtilis* 68 was observed in the presence of sucrose (Zherebtsov et al. 2002). All these dissimilar results suggest that there is no uniform regulation mechanism for the production of microbial inulinases.

Very interesting results for inulinase production have been obtained with complex substrates from agro-industrial wastes or vegetal extracts. Among these plant materials dahlia, Jerusalem artichoke, garlic, and others have been cited in the literature (Trivedi et al. 2012). In the present case, when yacon juice was used as CES in *A. kawachii* cultures, the highest enzymatic activity (35 mU x ml^{-1}) occurred at 78 hrs of culture. Meanwhile, when food grade inulin was used as CES, the maximum activity (20 mU x ml^{-1}) was achieved at 64 hrs culture. These culture times were comparable to those reported for other inulinase producing fungi: 72 hrs for *Penicillium sp*. and 110 hrs for *Aspergillus niger* (Parekh and Margaritis, 1985). Manzoni and Cavazzoni (1992) reported a similar difference between pure inulin and natural extracts in the production of an extra-cellular inulinase. Those authors studied the production of inulinase with four yeast strains in pure inulin and Jerusalem artichoke extract. In inulin, the highest production of inulinase was 2.8 U x ml^{-1} using *Candida pseudotropicalis*, whereas substantially higher inulinase activities were achieved when a Jerusalem artichoke extract was used: 14.6 U x ml^{-1} for *Candida kefyr*, 18.7 U x ml^{-1} for *C. pseudotropicalis*, 18.4 U x ml^{-1} for *Kluyveromyces marxianus var. bulgaricus*, and 14.3 U x ml^{-1} for *K. fragilis*. In dry Jerusalem artichoke extract higher inulinase production was obtained (96.6 U x ml^{-1}) after a seven-day fermentation, using *K. marxianus var. bulgaricus* (Manzoni and Cavazzoni, 1992). Moreover, a higher inulinase production was reported for *A. niger* using dandelion tap root extracts as a carbon source compared to pure chicory inulin (Kango, 2008).

In the present study, the highest enzyme activity was achieved using yacon juice as sole CES. As previously mentioned, a high percentage of yacon dry matter is composed by fructans, mainly short

chain oligomers (FOS), whereas the average chain length in food grade inulin is substantially larger (up to 60 monosaccharide units). The enzyme induction mechanism could be affected by the polymerization degree of these CES. In addition, the presence of natural promoting factors in yacon juice should be considered. Based on these considerations, yacon juice was chosen for further studies on *A. kawachii* inulinase production.

Effect of initial culture pH on inulinase production.

Cultures of *A. kawachii* in a minimal medium with yacon juice as CES were carried out at different initial pH values. For this purpose, the pH of the salt solution was adjusted to values from 3 to 6 prior to the addition of yacon juice, and pH of the complete medium was recorded. It should be mentioned that pH of yacon juice is 6.3. Results obtained indicate that the levels of inulinase activity achieved were strongly influenced by the initial pH of the culture. The highest value of inulinase activity (80 mU x ml^{-1}) was found at 78 hrs of cultivation when the initial pH of the culture was 3.5. A typical culture of *A. kawachii* at initial pH 3.5 is shown in Figure 2. Biomass concentration increased up to 24 hrs of cultivation, in parallel to pH change, a fact that was associated to the assimilation of nitrate as nitrogen source. At that time, around 90% of the initial reducing sugars had been consumed. Later on, during the stationary phase of the culture, inulinase activity increase substantially. The delay of enzyme production with respect to cell growth could be ascribed to the inhibition caused by simple sugars over inulinase synthesis. Inulinase activity increased with time up to 80 hrs of cultivation even tough reducing sugars were almost exhausted at ~50 hrs. In this case, enzyme synthesis could be supported by the assimilation of fructans remaining in the culture medium. Another possible explanation of this fact could be associated to the excretion of previously synthesized inulinase molecules from the fungal protoplasm into the culture media. Whichever the case, all further cultures were carried out at initial pH: 3.5.

Enzyme production in bioreactor

Cultures of *A. kawachii* were carried out in a 5 l-fermentor using yacon juice as CES at initial pH 3.5 (Figure 3). In this case, inulinase activity started to be noticeable when the concentration of reducing sugars was quite low in a similar way as above described for Erlenmeyer cultures. The maximum inulinase activity (110 mU x ml^{-1}) was reached at 40 hrs of culture. Enzyme productivity in bioreactor cultures was substantially higher than in Erlenmeyer flasks, probably due to a better performance in mixing and O_2 transfer.

Partial characterization of the enzyme

Enzyme stability. The crude inulinase preparation from *A. kawachii* was characterized in terms of its stability towards extreme pH and temperature. When studding thermal stability, the effect of the addition of some stabilizing agents was also evaluated.

As can be seen in Figure 4a, the enzyme is highly stable at pH values ranging from 3 to 9 and most of its activity is lost at pH values higher than 10. In addition, enzyme was stable for 3 hrs at temperatures from 37 to 60ºC (Figure 4b); while at 65ºC it exhibits a first order decay with a half life calculated in 75 min. When some additives were added to the crude filtrate, such as glycerol and propyleneglycol, the enzyme stability was slightly increased, especially at higher temperatures (data not shown). Nevertheless, the addition of such substances should be evaluated in terms of a cost/benefit analysis regarding the application of the enzyme, since the crude enzyme preparation is quite stable up to 60ºC.

The increasing interest in applying enzymes in industrial processes has supported the search for biocatalysts with new or improved properties. Due to the unique capacity of enzymes to catalyze reactions at high rate and specificity under a variety of conditions, the development of new biocatalytic processes is feasible and potentially profitable. The use of biotransformation in industry will increase and doubling of the number of industrially established biocatalytic processes every decade has been forecasted (Eijsink et al. 2005).

Unfortunately, the currently available enzymes are seldom optimally suited for industrial applications. This incompatibility often relates to the stability of the enzymes under process conditions. Although sometimes it is beneficial to adapt industrial processes to mild and environmentally benign conditions,

as those inherent to enzyme catalysis, the use of more extreme conditions is often desirable. For instance, the use of high process temperatures may be beneficial with respect to factors such as high substrate and product solubility, low viscosity, high process speed and no microbial contamination. Regardless of process conditions, the stability of the biocatalyst is often a key economic factor.

The study of temperature and pH effect on the activity of enzyme preparations is very important for their application. Low pH and high temperature offer advantages for industrial fructose syrup production from inulin, since high temperatures favour solubility of inulin allowing high substrate concentrations to be used, while also preventing microbial contamination (Vandamme and Derycle, 1983). Higher thermostability of industrially important enzymes brings down production costs (Saber and El-Naggar, 2009).

Effect of pH on inulinase activity

Inulinase from *A. kawachii* exhibits optimum activity at pH 3 with inulin as substrate (Figure 5), which is quite different from those previously reported for other inulinases. Optimum pHs of the purified inulinases from fungi and yeasts are in the range from 4.5 to 6.0 (Pandey et al. 1999; Singh et al. 2007a; Gong et al. 2008; Sheng et al. 2008). This feature makes this biocatalyst interesting from an industrial application perspective, since its acid stability may allow the development of reactions at strong acidic conditions.

Effects of metal ions on inulinase activity

Inulinase from *A. kawachii* is partially inhibited by K^{+}, Ca^{2+}, Hg^{+2}, Mn^{+2}, Zn^{2+} and Fe^{3+} at 1 mM concentrations (Figure 6). These results are quite different from those earlier observed for other microbial inulinases. Sharma et al. (2006) and Sheng et al. (2008) working with *Streptomyces* sp and *Cryptococcus aureus* respectively, reported the stimulation of inulinase activity by K^{+}, Na^{+}, Fe^{2+}, Cu^{2+} and Ca^{2+} and the complete inhibition by Hg^{2+}. The strong inhibitory effect observed with Hg^{2+} suggests that some -SH-group in the protein might be essential for activity.

Hydrolysis of inulin

Inulinase activity in the crude culture filtrate was analyzed in terms of its mode of action on inulin as substrate. Reaction products were analyzed by TLC (Figure 7). As shown in lanes 3-9, during the reaction time course fructose was liberated as the sole product, which indicates that this enzyme display a characteristic pattern of inulin hydrolysis by an exo-activity.

CONCLUDING REMARKS

As far as the authors are aware, this is the first report on inulinase production from *A. kawachii*. Yacon derived products, particularly yacon juice, added to the culture medium proved to be a good CES for fungal growth as well as an inducer of enzyme synthesis. Partial characterization of the enzyme revealed that it is quite stable in a wide range of pH and temperature. In addition, characterization of the reaction products revealed that this enzyme corresponds to an exo-type. These facts are promising considering its potential application in inulin hydrolysis for the production of high fructose syrups.

Purification and full biochemical characterization of this enzyme is under development as well as the corresponding gene cloning and over-expression in heterologous expression systems.

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Figures

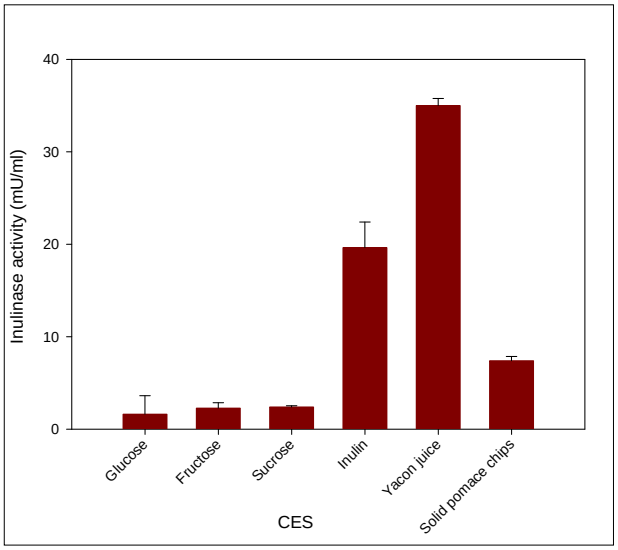

Fig. 1 Maximum inulinase activities produced by *A. kawachii* grown in different CES in Erlenmeyer flasks.

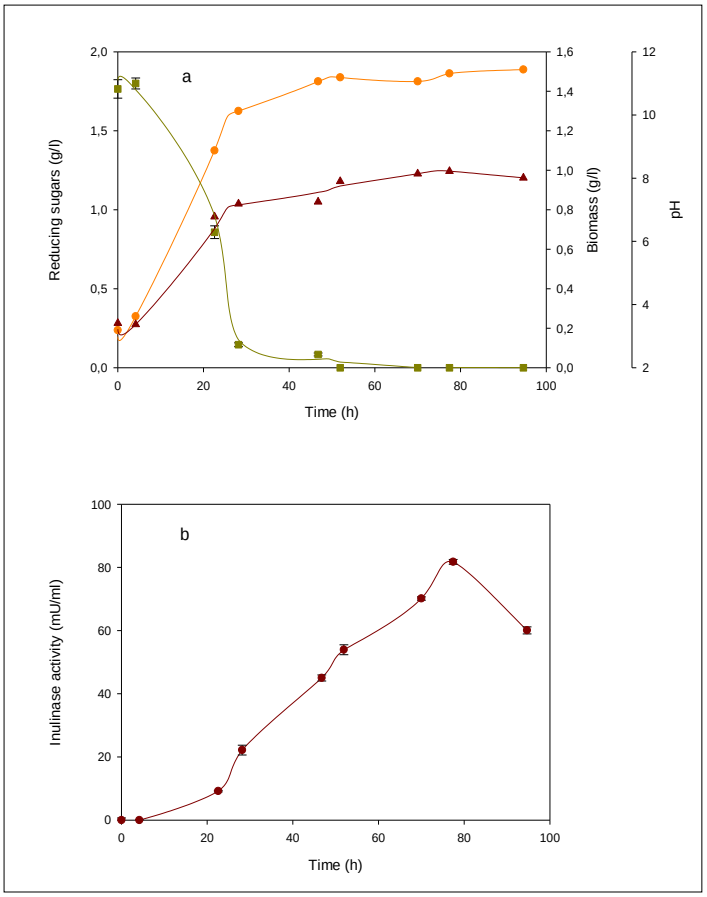

Fig. 2 Time course of *A. kawachii* culture using yacon juice as the sole CES in Erlenmeyer flasks. (a) (■) total sugars, (●) biomass concentration, (▲) pH. (b) (●) inulinase activity.

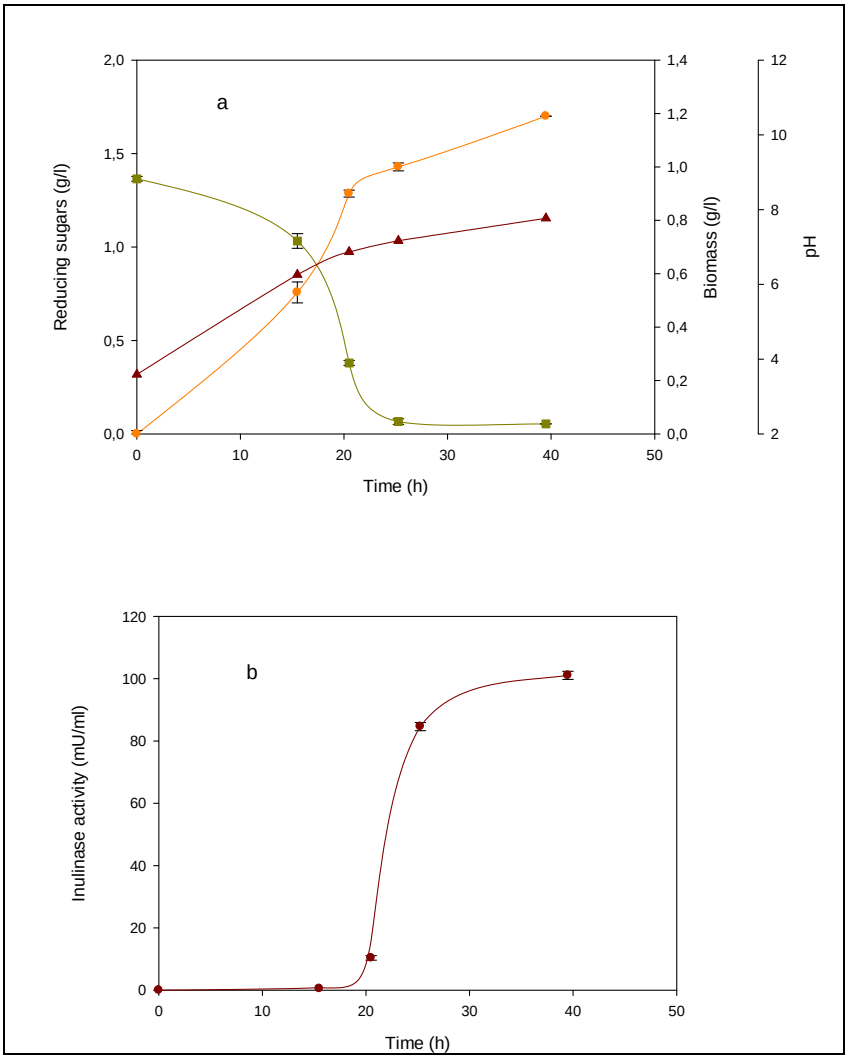

Fig. 3 Time course of bioreactor cultures of *A. kawachii* using yacon juice as the sole CES. (a) (■) total sugars, (●) biomass concentration, (▲) pH. (b) (●) inulinase activity.

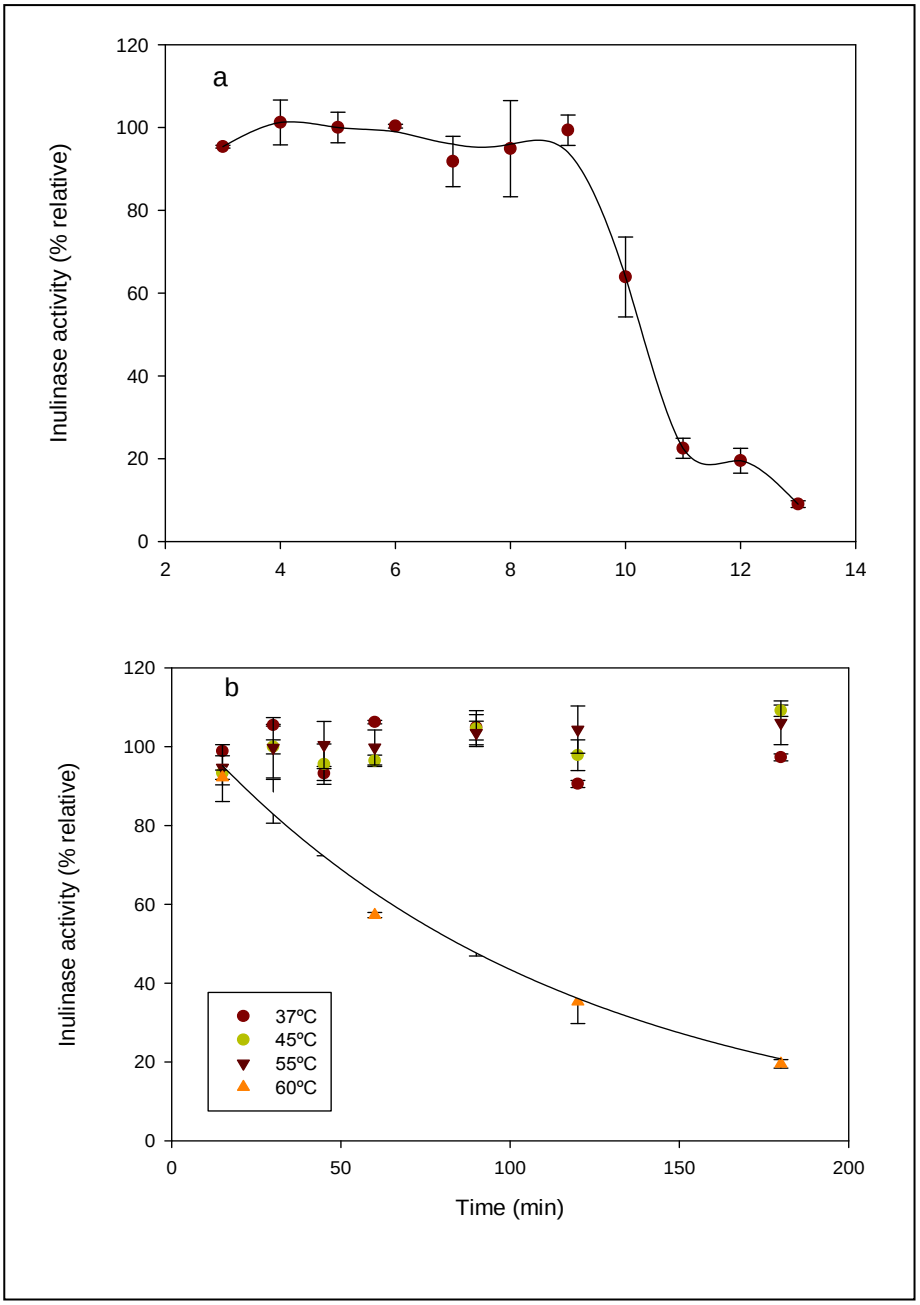

Fig. 4 (a) Inulinase pH stability at 37ºC min in Tris: Mes: Glycine buffer (0.02 M each pH values from 3-13); and (b) thermal stability at pH 4.0 at 37ºC; 45ºC; 55ºC; 60ºC. After incubation under different conditions, enzyme residual activity after 180 min was determined under standard conditions for enzyme activity assay.

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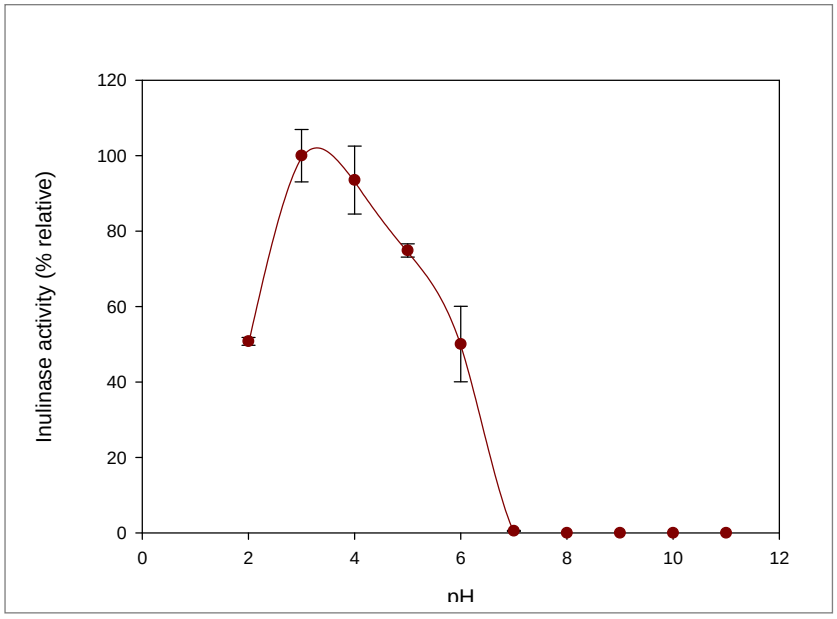

Fig. 5 Enzymatic activity profile of inulinase from *A. kawachii* at different pHs. The relative inulinase activity was determined using the standard assay conditions but varying the pH of the buffer (Tris:MES:Glycine, 20 mM each).

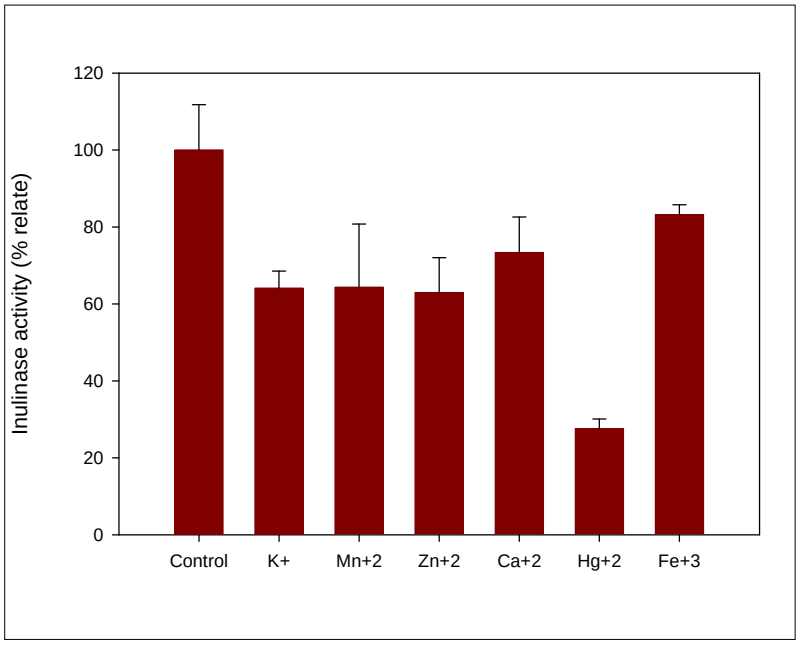

Fig. 6 Effects of metal ions on inulinase activity.

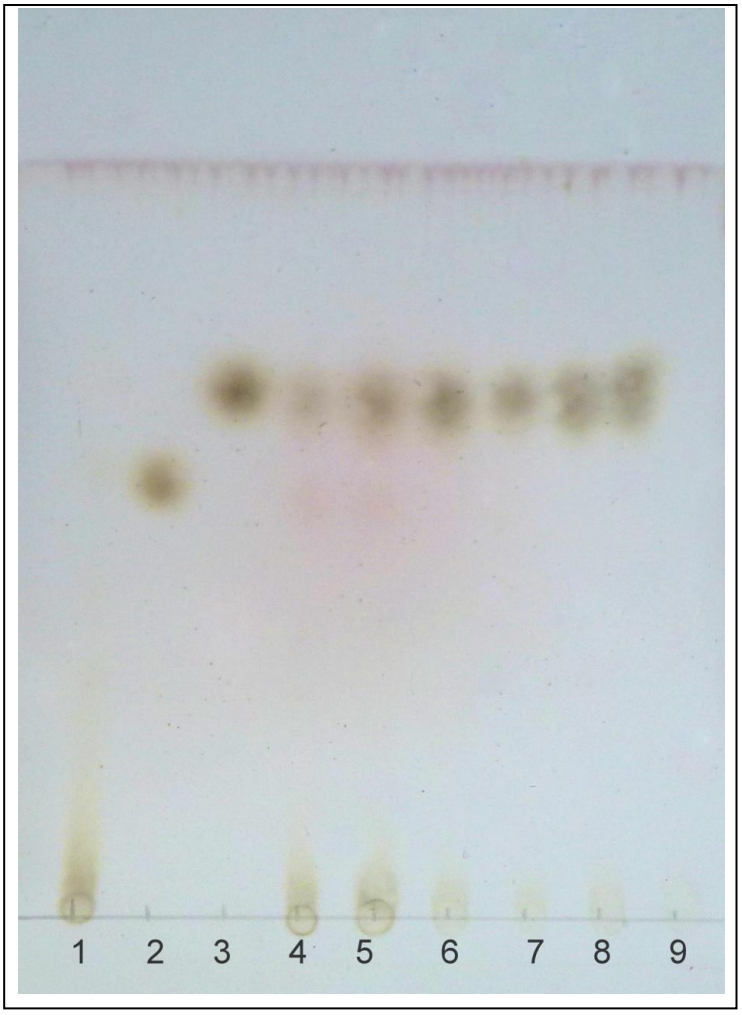

Fig. 7 TLC chromatogram of the products of hydrolysis of inulin with inulinase from *A. kawachii*. Lane 1: inulin standard, Lane 2: sucrose standard, Lane 3: fructose standard, Lanes 4-9: hydrolysis products of inulin by inulinase (reaction times from 1 to 6 hrs). Sample volume: 5 µ. TLC plates were developed twice with n-butanol-isopropanol-acetic acid–water (7:5:4:2) as the developing solvent in ascending TLC.

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