

Inulinase Production by a Mexican Semi-Desert Xerophylic *Penicillium citrinum* Strain under Submerged Culture

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Abstract: The aim of the study was to produce inulinase under submerged culture (SmC) by a xerophylic fungal strain isolated from the Mexican semi-desert and to verify its potential as an industrial inulinase producer. This enzyme can be obtained from microorganisms that live in close association with inulin plant store tissues. Inulin is a widespread plant polyfructan that serves as a storage polysaccharide in several plants and its depolymerization involves the action of inulinase. Inulinases are classified among hydrolases and target on the β -(2,1)-linkage of inulin which is hydrolyzed into fructose and glucose. These carbohydrates are promising candidates to be used as food ingredients and in production of fermenting sugars. However, their utilization is limited due to the high cost of their production thus it is necessary to find new inulinase producer strains with high productivity and desirable characteristics for industrial production. In the present study, *Penicillium citrinum* ESS produced extracellular inulinase with the highest enzymatic activity (18.7 U/L) at 48 h, 30°C and 25 rpm, respectively. Moreover, the medium that we propose for inulinase production has a simpler chemical composition than other reported in previous works. Even further improvements on the process are needed, within the experimental limits of this study, it was possible to demonstrate that the xerophylic fungal strain *Penicillium citrinum* ESS isolated from Mexican semi-desert could compete favorably with other fungal strains reported in literature to produce inulinase industrially.

Key words: Inulin, inulinase, Mexican semi-desert, *Penicillium citrinum* ESS, submerged culture, xerophylic

INTRODUCTION

Inulin is a widespread plant polyfructan that has linear chains of β -(2,1)-linked fructose residues attached to a terminal sucrose residue (Edelman and Jefford, 1964). This polyfructan serves as a storage polysaccharide in the Compositae and Gramineae families, and is accumulated in the underground parts of several plants of Asteracea, including rusby (*Vernoniaherbacea*), Jerusalem artichoke (*Helianthus tuberosus*) and chicory (*Cichoriumdivia*) (Vandamme and Derycke, 1983).

It has been previously described that organisms living in close association with plant tissues could be inulin degraders through enzymatic hydrolysis (Ohta *et al.*, 2004; Hyde and Soyong, 2008). This process involves the action of inulinases, which are classified among the hydrolases and target on the β -(2,1)-linkage of inulin and hydrolyze it into fructose and glucose. The general reaction mainly involves action of two enzymes: exo- and endoinulinase (Chi *et al.*, 2009) and potential biotechnological applications to these enzymes have been noticed (Ohta *et al.*, 1993).

These enzymes have been isolated from different microorganisms including *Penicillium* (Onodera and Shiomi, 1992; Balayan *et al.*, 1996; Pessoni *et al.*, 1999), *Kluyveromyces* (Gupta *et al.*, 1994) and *Aspergillus* (Ettalibi and Baratti, 1990; Arand *et al.*, 2002). In recent years, our group reported xerophilic fungal strains isolated from the Mexican semi-desert which have demonstrated to present very efficient enzymatic machinery (Cruz-Hernandez *et al.*, 2005, 2006). These fungal strains have been studied due to their tolerance to extreme conditions typical from the region (long drought periods, high midday temperatures, and low night temperature, high osmotic pressures). The aim of this research was to study inulinase production by a fungal strain isolated from Mexican semi-desert and to verify its potential as an industrial inulinase producer.

MATERIALS AND METHODS

Microorganism: *Penicilliumcitrinum*ESS was isolated from the Mexican semi-desert region (Cruz-Hernandez *et al.*, 2005). Strain spores were propagated, harvested in

a cryoprotector system and stored at -20°C. The strain was activated in Potato Dextrose Agar (PDA) and incubated at 30°C for 72 h; spores were suspended in Tween 80 (0.01%) and counted in a Neubauer chamber.

Culture conditions: Culture medium was prepared with the following composition (g/L): NaNO₃ (7.65), KH₂PO₄ (3.04), MgSO₄ (1.52), KCl (1.52) and inulin as a sole carbon source (from Dahlia tubers, Fluka ®) (5.0); pH value was adjusted to 5.5. Erlenmeyer flasks (500 mL) containing 100 mL of medium were inoculated with 1.0×10⁷ spores/mL and incubated (30 °C, 96 h) in a rotatory shaker (250 rpm). Samples (three individual flasks) were taken at 12 h intervals, and the mycelium was separated by filtration (Whatman No. 41) and the filtrate was considered as the enzymatic extract.

Protein determination: Total protein content was determined in the enzymatic extract according to the Bradford (1976), method using bovine serum albumin as protein standard. All assays were performed in triplicate for each analyzed flask.

Inulinase assay: The reaction mixture (0.5 mL) contained 10 g/L of inulin (Fluka) in 0.1 M sodium acetate buffer pH 5.0. After addition of the enzyme solution, the mixture was incubated at 37 °C for 12 h. The reaction was stopped by addition of 2 mL of Somogyi reagent (Somogyi, 1952). Then 1.5 mL of water was added and the mixture incubated at 100°C for 15 min. After cooling, 1 mL of Nelson reagent (Nelson, 1944) was added. The absorbance at 550 nm was measured with a Thermo Spectronic spectrophotometer against a blank containing only the acetate buffer. The amount of reducing sugars was estimated by comparison with a calibration curve made with fructose. One inulinase unit was defined as the amount of enzyme liberating one micromole of fructose equivalent per minute under the tested conditions.

Activity on sucrose: The reaction mixture (0.5 mL) contained 50 mM sucrose in a 0.1 M sodium acetate buffer pH 5.0. After addition of the enzyme solution, the mixture was incubated for 10 min at 30 °C the reducing sugars were estimated by the Somogyi-Nelson method as described for the determination of inulinase activity. The calibration curve was made with an equimolar solution of glucose and fructose. An invertase unit was the amount of enzyme which catalyzed the hydrolysis of one micromole of sucrose per minute.

RESULTS AND DISCUSSION

In the present study, *Penicilliumcitrinum*ESS inulinase production was kinetically evaluated using SmF with inulin as a sole carbon source. The maximal enzymatic production was obtained at 48 h, 30 °C and 250

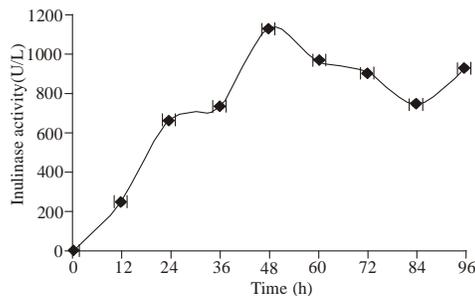


Fig. 1: Enzymatic activity during kinetics for inulinase production using *Penicilliumcitrinum*ESS

rpm for inulinase (18.7 U/L). After maximal enzymatic production was reached (48 h), enzyme value decreased until 15 U/L (96 h), probably due to proteolytic activity. In other study, maximum inulinase level (80 U/L) was reached at 60 h at 30 °C by *Aspergillusniger*; thereafter, enzyme activity decreased to about 28 U/L after 120 h (Poorna and Kulkarni, 1995).

Different optimal shaking speeds have been reported. Skowronek and Fiedurek (2006) reported as optimal shaking speed for inulinase biosynthesis by *A. niger* 20 Osm mycelium immobilized on pumice stones 200 rpm, and maximum inulinase yield was obtained after 60 h of growth in shaken cultures of *A. niger* van Teighem at 150 rpm (Poorna and Kulkarni, 1995).

The knowledge of temperature and pH influence on the activity of enzyme preparations is very important in food processes. The determination of optimum temperature and pH rate is particularly essential in enzymatic processes. High temperature and low pH may decrease the risk of contamination, improve the solubility of some substrates, including inulin, and can reduce the color formation in some syrups (Vandamme and Derycke, 1983). In the present study, pH values were into the acidic range during fermentation (5.8-5.9). This pH range has been reported for inulinase production using *A. niger* (Wallis *et al.*, 1997; Cruz *et al.*, 1998) and *P. janczewskii* (Pessoni *et al.*, 2002, 2007). As pH value diverged from the optimum level, the efficient functioning of enzyme could be affected due to the change in active site conformation which is determined, in part, by ionic and hydrogen bonding that can be affected by pH.

The increase in enzymatic activity (Fig. 1) was not usually associated with higher protein content in the culture medium (Fig. 2). The highest enzymatic activity for inulinase was not directly related to protein content. Highest enzymatic activity was observed at 48 h, while at 24 h it was observed the highest protein content in medium. Therefore, we speculate that increased enzymatic activity may be caused either by an increased fraction of native enzyme in total protein secreted into the medium or by a change in the enzyme itself. Another possibility is a different rate of utilization of medium proteins by strains of *Penicillium*.

Table 1: Comparison of inulinase activities obtained by different researches under different conditions

Microorganism	Carbon source	Incubation time (h)	Inulinase activity (U/L)	References
<i>A. niger</i> 20 Osm	Sucrose	96	22.10	Skowronek and Fiedurek (2006)
<i>A. niger</i> 13/36	Inulin	72	17.54	Skowronek and Fiedurek (2003)
<i>A. niger</i>	Sucrose, corn steep liquor	168	48.40	Ohta <i>et al.</i> (1993)
<i>A. niger</i>	Inulin, corn steep liquor	96	6.00	Poorna and Kulkarni (1995)
<i>A. niger</i>	Maltose, corn steep liquor	108	20.10	Derycke and Vandamme (1984)
<i>P. spinolosum</i>	Inulin	24	1.25	Ertan <i>et al.</i> (2003)
<i>P. purpurogenum</i>	Inulin, maltose	72	4.45	Onodera and Shiomi (1988)
<i>F. oxysporum</i>	Inulin	216	0.08	Gupta <i>et al.</i> (1990)
<i>P. citrinum</i> ESS	Inulin	48	18.70	Current study

Table 2: I/S ratio values from *penicilliumcitrinum* ESS inulinase

Time (h)	Specific activity (U*g/min)		I/S ratio
	Inulin	Sucrose	
12	8.77	4.46	1.96
24	12.86	6.34	2.02
36	15.83	7.65	2.06
48	24.37	12.59	1.93
60	24.63	12.89	1.91
72	1377.27	710.36	1.93
84	143.19	84.14	1.70
96	35.69	21.05	1.69

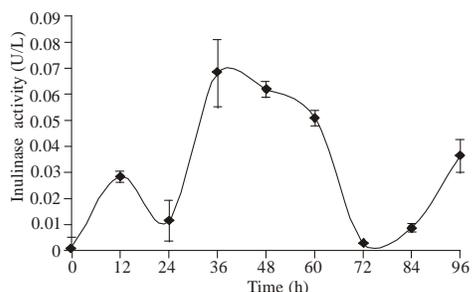


Fig. 2: Protein content during kinetics for inulinase production using *Penicilliumcitrinum*ESS

The results of this study were compared with values for inulinase production reported in the literature. The activity of inulinase produced by *Penicilliumcitrinum* ESS was within the values reported for *Aspergillus niger* (Derycke and Vandamme, 1984; Ohta *et al.*, 1993; Poorna and Kulkarni, 1995; Skowronek and Fiedurek, 2003, 2006) but significantly higher in comparison with the enzyme synthesized by *Penicillium purpurogenum* (Onodera and Shiomi, 1988; Ertan *et al.*, 2003) and *Fusariumoxysporum* (Gupta *et al.*, 1990) (Table 1). Even higher levels of inulinase activity are achieved by *Aspergillus niger*, the fungal strain tested in current work (*Penicilliumcitrinum* ESS) achieved a significant reduction in process time accompanied by a high level of inulinase activity comparable with these of *Aspergillus niger*, giving thus, the highest productivity.

Usually, inulinases are classified as 2, 1-β-D-fructanfructano hydrolase (EC 3.2.1.7) but the difference with invertases (EC 3.2.1.26) is not clear for the microbial enzymes due to they have a wide substrate specificity. It is generally accepted that the ratio of the activity on inulin versus sucrose (I/S ratio) classifies the enzymes: for inulinase I/S ratio is greater than 1, while for sucrose is lower (Rouwenhoirst *et al.*, 1990; Qang *et al.*, 1999). It

was shown that the inulinase produced in the current work was active on inulin and sucrose. The ratio of these two activities (I/S ratio) varied from 1.69 to 2.06 during fermentation process. Thus, the enzyme was confirmed as inulinase. In Table 2, specific activities on inulin and sucrose, and I/S ratios are shown.

The enzyme world market is around one billion dollars per year, with an enhancement on amount of patents and research in this area. Many enzymes are produced by submerged fermentation and are considered commodities, since their production and recovery costs are inversely proportional to their concentration in the fermented broth (Dyaz-Godynez *et al.*, 2001). Inulinases are promising candidates for use as complements in food ingredients and in the production of fermenting sugars. However, their utilization has been limited due to production costs.

On the other hand, it has been also reported that inulinase production is influenced by factors such as the presence of metal ions. Although more research is needed, it was observed a positive influence of KCl (0.01M), MgSO₄ (0.01M), and FeSO₄ (0.001M). Additionally, Fiedurek and Gomada (2000) described the dissolved oxygen as one of the important parameters of aerobic fermentation processes. Critical DO concentration to determine the optimum capability of a microorganism varies and depends on the desired end product. Oxygen limitation in the culture medium may depend on mycelium density, carbon source concentration and pH value. Skowronek and Fiedurek (2006) achieved a six-fold higher productivity of inulinase from *Aspergillus niger* Osm during conventional aeration in comparison with unconventional oxygenation culture at 96 h. Besides, fermentation was quite resistant to contamination due to the presence of hydrogen peroxide.

Nowadays, thermophiles and especially thermophilic enzymes have gained a great deal of interest both as analytical tools, and as biocatalysts for application on a large scale. Thermophilic fungi can produce inulinases of higher optimum temperature which is an extremely important factor in the commercial industrial production of fructose or fructo oligosaccharides from inulin. This is mainly because high temperatures ensure proper solubility of inulin and also prevent microbial contamination (Gao *et al.*, 2009; Turner *et al.*, 2007; Ettalibi and Baratti, 2001; Vandamme and Derycke, 1983). Moreover, higher thermostability of the industrially important enzymes also

brings down the cost of production because lower amounts of enzyme are required to produce the desired product (Gill *et al.*, 2006). Thus, inulinase productivity values from *Penicillium citrinum* ESS could be enhanced, varying parameters such as incubation temperature, aeration and media composition.

CONCLUSION

Even further improvements on the process are needed, within the experimental limits of this study, it was possible to demonstrate that the xerophylic fungal strain isolated from Mexican semi-dessert (*Penicillium citrinum* ESS) could compete favorably with other fungal strains reported in literature to produce inulinase industrially. Moreover, the medium we propose for inulinase production has a simple chemical composition in comparison with the complex media reported by other authors.

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