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Production of Inulinases from *Penicillium spinulosum*, *Aspergillus parasiticus* NRRL2999 and *Trichoderma viride*

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Abstract: The some cultivation conditions of fungal inulinases and action of enzymes were studied in containing Jerusalem artichoke powder. The highest inulinase activity from *A. parasiticus* observed at 30°C, the initial pH at 5.0 and cultivation time was 24th hours. *P. spinulosum* inulinase activity was maximum when the cultivation time was 2 days at a temperature of 30°C with the initial pH at 5.0. The maximum inulinase activity of *T. viride* was determined at 25°C, the initial pH at 6.0 and cultivation time was 4 days. It was found that *A. parasiticus* and *P. spinulosum* had exoinulinase however *T. viride* had endoinulinase by TLC (Thin Layer Chromatography). The maximum I/S ratios for *A. parasiticus*, *P. spinulosum* and *T. viride* were 1.61; 1.79 and 2.6 under optimal conditions, respectively. *A. parasiticus* and *P. spinulosum* could be used as source of exoinulinase moreover *T. viride* could be used as source of endoinulinase.

Key words: Inulinase, production, fungi

Introduction

Inulin is a linear β (2-1) linked fructose polymer with a terminal glucose unit that can be found as reserve carbohydrate compositae family plant tubers. (Vullo *et al.*, 1991; Kato *et al.*, 1999). Inulin is used as raw material on the production of ultra high fructose syrup, ethanol, acetobutanol, gluconic acid, sorbitol and fructooligosaccharides. Nakamura *et al.* (1997) inulin is hydrolysed by inulinase (2,1- β -D fructan fructanohydrolase EC. 3.2.1.7). Inulinases have been produced and purified from yeast, moulds and bacteria and have potential uses in the preparation of fructose syrups from inulin and invert sugar in the food industry. Kushi *et al.* (2000). As many microbial preparations of inulinase possess remarkable invertase (S) activity accompanying the inulinase activity (I), their catalytic activity is described in terms of I/S or S/I ratios. Pessoni *et al.* (1999) Inulinases from plants are not active on sucrose, although inulin is strongly attacked. On the other hand, inulinases found in microorganisms are classified in two types by the mode of action on inulin. One, endoinulinase, produces oligosaccharides and the other, exoinulinase, liberate fructose. Onodera and Shiomi (1992). Microbial inulinases are an important class of industrial enzyme that have gained much attention recently. Inulinases have been characterised from inulin-storing tissues of plants but their quantity is not enough to be exploited for commercial production of fructose from inulin (Gupta *et al.*, 1994; Pandey *et al.*, 1999). Microbial inulinases are potentially useful for the preparation of high fructose syrups from inulin. (Ettalibi and Baratti, 1987; Pessoni *et al.*, 1999).

In this study, we researched the production conditions of inulinase enzymes from *Penicillium spinulosum*, *Aspergillus parasiticus* NRRL2999 and *Trichoderma viride* and the determination of hydrolysis products of inulin with crude enzyme by TLC.

Materials and Methods

Fungi: Fungi were obtained from Hacettepe University, Faculty of Science, Department of Biology, Division of Biotechnology, Beytepe, Ankara-TURKEY. The organisms were maintained on PDA slants.

Preparation of Jerusalem artichoke: *Jerusalem artichokes* were washed with cold water, sliced with blender and than dried in Pasteur oven at 80°C. The dried slices were milled to a fine powder with mill (Öngen-Baysal *et al.*, 1994).

Medium and cultivation: The medium for enzyme production contained 3.0% *Jerusalem artichoke* powder, 0.23% NH_4NO_3 , 0.37% $(\text{NH}_4)_2\text{HPO}_4$, 0.1% K_2HPO_4 , 0.05% MgSO_4 , 0.15% yeast extract. Medium was autoclaved at 115°C for 30 min. Spores were suspended with 0.9% NaCl and 1 ml of this was used to inoculate 250 ml flasks. Flasks containing 50 ml medium were incubated at 30°C on orbital shaker at 200 rpm (Öngen-Baysal *et al.*, 1994).

Crude enzyme: From production medium the mycelia was collected by filtration using Whatman No 1 filter paper. The culture filtrate was centrifuged at 5000 rpm for 10 min. The supernatants were used as the crude enzyme

throughout the experiments for the enzyme assays (Xiao *et al.*, 1988; Nguyen *et al.*, 1999).

Enzyme assays

Inulinase and invertase activity: Enzyme assays were measured by the 3, 5-Dinitrosalicylic acid method Miller (1959) and carried out according to Haraguchi *et al.* (1990). The amount of reducing sugar was estimated by comparison with a calibration curve was made with fructose for inulinase activity. The calibration curve was made with an equimolar solution of glucose and fructose for invertase activity.

One unit of inulinase activity was defined as micromole of fructose produced per minute by 1 ml of cell free medium. One unit of invertase was defined micromole of reducing sugar produced per minute by 1 ml of cell free medium.

Determination of hydrolysis products: The hydrolysis products of inulin with crude enzyme were determined by thin-layer chromatography (TLC) using 20 cm plates. (Merck, TLC aluminum sheets 20x20 cm, Silica gel 60 F₂₅₄). Azhari *et al.*, (1989) A mixture of acetic acid: chloroform: water. (35:30:5, v/v/v) was used as the developing solvent. On the air-dried plates, carbohydrates were detected by staining with aniline-diphenylamine reagent. This reagent was prepared by mixing a solution of 1 ml of aniline and 1 g of diphenylamine in 100 ml acetone, with 10 ml 85% phosphoric acid; the latter component was added just prior to use. The plate was sprayed with the staining reagent and colour developed after heating for 15 min. at 120°C. By using this procedure, fructose and fructoseoligomers were visualised as brown spots, glucose as a blue spot and sucrose as a dark green spot. The colours are clearly visible for up to 6 h (Azhari *et al.*, 1989).

Results

Effect of cultivation time: Table 1, the maximum inulinase activity (I/S_{max}=1.13) of *P. spinulosum* was found at 24 hours of cultivation period. It was determined that *A. parasiticus* inulinase activity was maximum (I/S_{max}=1.61) at 2nd day and inulinase activity of *T. viride* was maximum (I/S_{max} = 2.0) at 4 th day (Table 1).

Effect of initial pH: The initial pH of culture medium was adjusted to different values with 1 N HCl or NaOH solution. The *A. parasiticus* and *T. viride* inulinase activities were the highest (I/S_{max}=1.31; I/S_{max}=2.0, respectively) when the initial pHs were adjusted to 6.0. For *P. spinulosum* inulinase activity this pH value was 5.0 (Table 2).

Table 1: Effect of cultivation time

Time (Days)	Mycelia weight (g dry wt 50 ml)	Inulinase activity (Uml) (I)	Invertase activity (Uml) (S)	I/S
<i>A. parasiticus</i>				
1	0.34	2.9	2.2	1.31
2	0.56	1.6	1.0	1.61
3	0.67	1.1	1.1	0.90
4	0.99	0.7	0.8	0.87
<i>P. spinulosum</i>				
1	0.75	1.25	1.10	1.13
2	0.80	1.60	1.60	1.0
3	0.83	0.80	0.80	1.0
4	0.81	0.40	0.48	0.85
<i>T. viride</i>				
1	0.33	0.67	0.38	1.76
2	0.42	0.75	0.48	1.56
3	0.58	0.80	0.47	1.70
4	0.61	0.94	0.47	2.00
5	0.64	0.90	0.47	1.91
6	0.65	0.90	0.52	1.73

Table 2: Effect of initial pH

Initial PH	Mycelia weight (g dry wt 50 ml)	Inulinase activity (Uml) (I)	Invertase activity (Uml) (S)	I/S
<i>A. parasiticus</i>				
4	0.65	1.70	3.77	0.45
5	0.79	1.77	1.88	0.94
6	0.82	2.90	2.20	1.31
7	0.77	2.40	2.00	1.20
<i>P. spinulosum</i>				
4	0.69	1.05	0.77	1.36
5	0.78	1.45	0.81	1.79
6	0.88	1.65	1.62	1.01
7	0.81	1.15	1.83	0.62
<i>T. viride</i>				
4	0.64	0.98	0.58	1.68
5	0.63	0.89	0.55	1.61
6	0.68	0.94	0.47	2.00
7	0.43	0.00	0.00	-

Table 3: Effect of cultivation temperature

Temperature (°C)	Mycelia weight (g dry wt 50 ml)	Inulinase activity (Uml) (I)	Invertase activity (Uml) (S)	I/S
<i>A. parasiticus</i>				
25	1.06	1.25	1.36	0.91
30	0.83	2.90	2.00	1.45
35	0.80	2.70	3.30	0.81
40	0.79	2.10	4.00	0.52
<i>P. spinulosum</i>				
25	0.80	1.01	1.08	0.93
30	0.85	1.67	1.45	1.15
35	0.76	1.38	2.00	0.65
40	0.72	1.58	2.71	0.57
<i>T. viride</i>				
25	0.67	0.80	0.31	2.60
30	0.63	1.09	0.47	2.31
35	0.59	0.96	0.52	1.84
40	0.52	1.18	2.20	0.53

Effect of cultivation temperature: Effect of cultivation temperature on the enzyme activity was shown in Table 3. The temperature in which the highest I/S ratios measured were 25°C and 30°C.

Determination of hydrolysis products: The evaluation of TLC results was carried out according to Ettalibi M. and

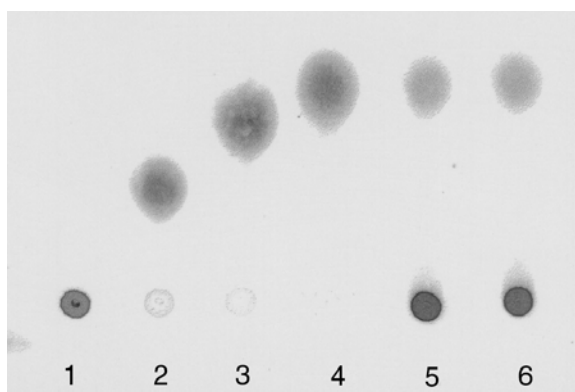


Fig. 1: Determination of hydrolysis products of inulinase from *A. parasiticus* (TLC) 1: Inulin (St) 2: Sucrose (St) 3: Glucose (St) 4: Fructose (St) 5: 10 min. hydrolysis 6: 30 min. hydrolysis

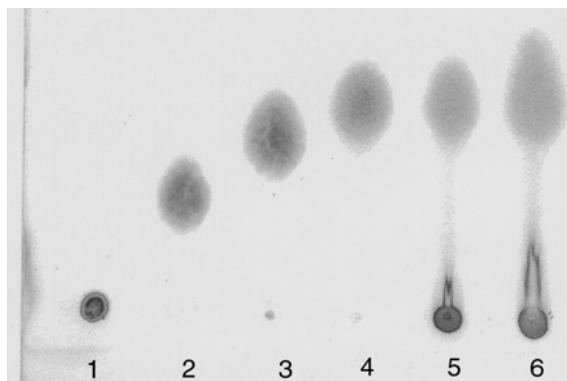


Fig. 2: Determination of hydrolysis products of inulinase from *P. spinulosum* (TLC) 1: Inulin (St) 2: Sucrose (St) 3: Glucose (St) 4: Fructose (St) 5: 10 min. hydrolysis 6: 30 min. hydrolysis

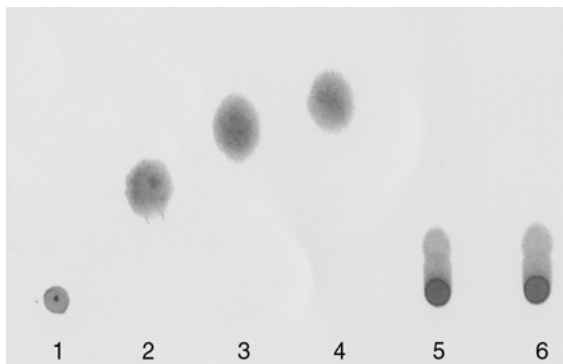


Fig. 3: Determination of hydrolysis products of inulinase from *T. viride* (TLC) 1: Inulin (St) 2: Sucrose (St) 3: Glucose (St) 4: Fructose (St) 5: 10 min. hydrolysis 6: 30 min. hydrolysis

Baratti (1987) Azhari *et al.* (1989) and Haraguchi *et al.* (1990). According to TLC results, it was determined that *A. parasiticus* and *P. spinulosum* inulinases are exoinulinase because their hydrolyse product is only free fructose. (Fig. 1-2, respectively) Fructooligomers were produced from the *T. viride* inulinase reaction. Thus, *T. viride* inulinase is an endo-inulinase. (Fig. 3).

Discussion

In this study, the inulinase activities and I/S ratios of three fungi were determined depending on the cultivation time (Table 1) This period was determined as 72 hours for *Penicillium* sp. according to Derycke and Vandamme (1984). Also this period was defined 40 hours (I/S= 1.46) for *Penicillium rugulosum* Barthomeuf *et al.* (1991), seven days at 27°C for *Chrysosporium pannorum* Xiao *et al.* (1988) and 24 hours for *Kluyveromyces marxianus* (Parekh and Margaritis, 1985). It was not discussed whether short or long production period have advantage or not in the previous studies. Generally maximum activity value depending on cultivation time and cultivation condition were investigated. Moreover, it was also obtained difference at cultivation time in the studies involving same genus. (Derycke and Vandamme, 1984; Barthomeuf *et al.*, 1991).

The highest I/S ratios of *A. parasiticus* and *T. viride* were found at pH: 6 when the effect of initial pH on the enzyme activity was researched. For *P. spinulosum* this pH value was 5.0. Similar results were explained in the previous studies: For *K. marxianus* pH 5.6 Rouwenhorst *et al.* (1990), for *C. pannorum* pH: 4.5-5.5 Xiao *et al.* (1988), for *Bacillus* sp. pH: 7.0-8.5 Allais *et al.* (1987), for *A. globiformis* pH: 7.0 Haraguchi *et al.* (1990), for *P. rugulosum* pH: 5.0-6.0 Barthomeuf *et al.* (1991) and for *F. oxysporum* pH: 5.5. Gupta *et al.* (1990). Generally, it was observed that the fungi preferred pH: 5.0-6.0 for inulinase production. Similarly in this study the fungi were determined to prefer pH 5.0-6.0 for inulinase production.

In the studies related to the effects of cultivation temperature on enzyme activity, the highest I/S ratios were: for *C. pannorum* 27°C Xiao *et al.* (1989), *F. oxysporum* 25°C Gupta *et al.* (1990), *K. marxianus* 35°C Rouwenhorst *et al.* (1990) and for *Penicillium* sp. 30-33°C for *A. niger* 28°C (Derycke and Vandamme 1984). In this study the findings of temperature parameters were similar with the previous findings.

When examined by hydrolysis products it was observed that the hydrolysis products of the inulinase *A. parasiticus* and *P. spinulosum* were only free fructose, but *T. viride* inulinase was produced short chained fructooligosaccharides. According to these results, it was determined that *A. parasiticus* and *P. spinulosum* inulinases were exoinulinase, *T. viride* inulinase was endo inulinase (Fig. 1-3).

Exoinulinases split off terminal fructose units successively from non-reducing end of the inulin molecule. Thus, exoinulinases are used for the production of high fructose syrup. Endoinulinases are successful in the production of fructooligosaccharides, excluding

exoinulinase activity Kim *et al.* (1997). *A. parasiticus*, *P. spinulosum* and *T. viride* have strongly inulinase activity. It was reported the source of inulinase was particularly *Penicillium* and *Aspergillus* species in previous studies. It was determined to aim of using of the enzymes from *A. parasiticus*, *P. spinulosum* and *T. viride* in the industrial area and we suggested that it could be obtained more definite results for industrial use with more detailed investigations such as purification, thermal and pH stability.

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