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Determination of Optimum Cultivation Conditions on the Production of Inulinase from *Rhizoctonia solani*

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Abstract: *Rhizoctonia solani* isolated from Tekirdag-Turkey soil, was found to produce a very active inulinase enzyme. The optimum conditions for enzyme production were determined. Maximum growth was observed at 45°C while the highest inulinase production was determined at 40°C. The best inulinase production was observed at an initial medium pH 6.0 and on the first day of cultivation time. Inulin and Jerusalem artichoke powder as carbon source were the most effective on production of inulinase from *R. solani*. The highest productivity was recorded in the presence of 10 mM $NH_4H_2PO_4$+10 mM NH_4NO_3 as nitrogen source.

Key words: Inulin, inulinase, production, *Rhizoctonia solani*

Introduction

Inulin is a polysaccharide contained in dahlia, chicory, Jerusalem artichoke and so on. Its chemical structure is a linear β 2, 1-linked fructose polymer terminated by a sucrose residue. Haraguchi *et al.* (1990). Inulin has been considered a possible source for production of fructose either by acid hydrolysis or by enzymatic degradation of the polymer. Recently, inulin has been employed in diagnostic and has also been considered for use as a carrier in controlled delivery of drugs. Azhari *et al.* (1989) inulinases are enzymes that degrade the β-(2→1)-linkages of β-fructan chains by two mechanisms. Exo-inulinases (β-D-fructopyranoside fructohydrolase, E.C.3.2.1.80) sequentially split-off the terminal β-(2→1)-fructofuranosidic bonds, whereas endo-action enzymes (β-D-fructan fructonohydrolase, E.C. 3.2.1.7) hydrolyse the internal linkages in inulin to release a set of inulo-oligosaccharides as the main reaction products. Arand *et al.* (2002).

Many organisms, including filamentous fungi, yeast and bacteria are able to produce inulinase. Wei *et al.* (1999). The 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).

In this report, we described the optimal conditions for inulinase enzyme from *R. solani* growth on Jerusalem artichoke powder.

Materials and Methods

Microorganism: *Rhizoctonia solani* was isolated and identified in Trakya University, Faculty of Agriculture, Department of Plant Project from Tekirdag-Turkey soil. The screening procedure for *R. solani* was based on use of Jerusalem artichoke as the sole carbon source. A modification of the medium of Derycke and Vandamme was used. Derycke and Vandamme (1984). This led to identification of *R. solani* as the best inulinase producer.

Screening medium: The screening medium was modified according to Derycke and Vandamme and had the following composition: Jerusalem artichoke powder 1%; K_2HPO_4 1%; $MgSO_4.7H_2O$ 0.05%; $NaNO_3$ 0.15%; KCl 0.05%; $FeSO_4.7H_2O$ 0.01%; $NH_4H_2PO_4$ 0.2%; Agar 1.8%; initial pH was 6.0. *R solani* were plated on the agar medium and incubated at 28°C for 4 days.

Preparation of Jerusalem artichoke: Jerusalem artichokes were washed with cold water, sliced and then dried in Pasteur oven at 80°C. After milling, the resultant fine powder was used directly as a carbon source. Ongen-Baysal *et al.* (1994).

Medium and cultivation: The medium for the enzyme production contained Jerusalem artichoke powder 3.0%; NH_4NO_3 0.23%; $(NH_4)_2HPO_4$ 0.37%; K_2HPO_4 0.1; $MgSO_4.7H_2O$ 0.05%; yeast extract 0.15% in 50 ml of deionized water in a 250 ml erlenmeyer flask. The medium was autoclaved at 115°C for 30 min. After inoculation, the flask was incubated on a rotary shaker at different temperature and 200 rpm Ongen-Baysal *et al.* (1994).

Enzyme preparation: The mycelia was collected by filtration using Whatman No. 1 filter paper from production medium. The culture filtrate was centrifuged at 4000 x g for 10 min. (Hettich Zentrifugen EBA 21). The supernatants were used as the crude enzyme throughout the experiments for the enzyme assays (Xiao *et al.*, 1988; Nguyen *et al.*, 1999).

Enzyme assay: Inulinase activity: Enzyme solution (0.1 ml) was mixed with 0.1% inulin (1 ml) in 0.1 M sodium acetate buffer pH 5.0 and then the mixture was incubated at 35°C for 15 min. As a result of reaction, reducing sugar was determined by the 3, 5 dinitrosalisilic acid method. Miller (1959). One unit inulinase activity was defined as micromole of fructose produced per minute by 1 ml of enzyme solution at 35°C and pH 5.0.

Invertase activity: The reaction mixture contained 0.1 ml of enzyme solution and 1 ml of 0.1% sucrose in 0.1 M sodium acetate buffer pH 5.0. The reaction conditions were the same as those described for the assay of inulinase activity. One unit of invertase activity was defined micromole of reducing sugar produced per minute by 1 ml of enzyme solution at 35°C and pH: 5.0.

Estimation of dry mycelia weight: Samples collected from production medium were dried for 24 h in a drying oven at 80°C.

Results and Discussion

Determination of inulinase-producing *R. solani*: *R. solani* was grown on screening medium, containing Jerusalem artichoke powder as the sole carbon source. This result showed that *R. solani* was the inulinase producer.

Culture condition for inulinase production

Effect of cultivation time: As shown in Table 1, the maximum I/S ratio (I/S_{max} : 6.4) was found on the 2nd day of cultivation period at 30°C and pH: 5.0. This period was determined as 72 h for *Penicillium sp.* according to Derycke and Vandamme (1984). Also this period was defined 40 h (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 h for *Kluyeromyces marxianus*, Parekh and Margaritis (1985).

Effect of initial pH of the medium: The effect of medium initial pH was investigated at growth temperatures 30°C. Maximum I/S ratio (I/S_{max}: 17.7) was observed at initial pH: 6.0 (Table 2). Similar results were explained in the previous studies; *K. marxianus* pH 5.6, Rouwenhorst *et al.* (1990) *C. pannorum* pH: 4.5-5.5, Xiao *et al.* (1988) for *Bacillus sp.* pH: 7.0-8.5, Allais *et al.* (1986), for *A. globiformus* pH: 7.0, Haraguchi *et al.* (1990) for *P. rugulosum* pH: 5.0-6.0, Barthomeuf *et al.* (1991) and *F. oxysporium* pH: 5.5, Gupta *et al.* (1990). Generally, it was observed that the fungi preferred pH: 5.0-6.0 for inulinase production.

Table 1: Effect of cultivation time on inulinase production by *R. solani*

Days	Mycelial weight g dry wt 50 ml^{-1}	Inulinase (I) Uml^{-1}	Invertase (S) Uml^{-1}	I/S
1	0.40	0.784	0.056	14.00
2	0.41	1.792	0.280	6.40
3	0.60	1.791	0.952	1.88
4	0.65	1.344	1.120	1.19

Table 2: Effect of initial pH on inulinase production by *R.solani*

Initial pH	Mycelial weight g dry wt 50 ml^{-1}	Inulinase (I) Uml^{-1}	Invertase (S) Uml^{-1}	I/S
4	0.40	2.912	0.784	3.70
5	0.41	1.383	0.112	12.30
6	0.38	0.996	0.056	17.70
7	0.36	0.739	0.054	13.68

Table 3: Effect of temperature on inulinase production by *R. solani*

Temperature (°C)	Mycelial weight g dry wt 50 ml^{-1}	Inulinase (I) Uml^{-1}	Invertase (S) Uml^{-1}	I/S
25	0.41	2.03	0.364	5.57
30	0.40	1.49	0.110	14.08
35	0.63	1.37	0.0987	13.80
40	1.01	2.37	0.140	16.92
45	1.52	1.39	0.16	8.68

Table 4: Effects of carbon source on inulinase production by *R. solani*

Carbon source	Inulinase (I) Uml^{-1}	Invertase (S) Uml^{-1}	I/S
Inulin (1%)	1.932	0.367	5.264
Inulin (3%)	3.02	0.925	3.264
Jerusalem artichoke (1%)	1.328	0.112	11.857
Jerusalem artichoke (3%)	2.878	0.227	12.670
Soluble starch (1%)	0.084	0.082	1.024
Cellulose (1%)	0.086	0.00	-
Pectin (1%)	0.097	0.094	1.031
Maltose (1%)	0.060	0.00	-
Sucrose (1%)	0.27	0.230	1.173
Fructose (1%)	0.012	0.00	-

Table 5: Effect of nitrogen source on inulinase production by *R. solani*

Nitrogen source (mM)	Inulinase (I)Uml^{-1}	Invertase (S) Uml^{-1}	I/S	Mycelial Weight g dry wt 50 ml^{-1}
Control *	2.778	0.181	15.340	0.43
$NH_4H_2PO_4$ (0.1)	1.691	0.150	11.270	0.38
$NH_4H_2PO_4$ (0.05)	2.657	0.190	13.980	0.50
NH_4NO_3 (0.1)	2.416	0.210	11.500	0.42
NH_4NO_3 (0.05)	2.174	0.170	12.780	0.43
$(NH_4)_2HPO_4$ (0.1)	2.295	0.252	9.107	0.78
$(NH_4)_2HPO_4$ (0.05)	2.536	0.241	10.520	0.80
$(NH_4)_2SO_4$ (0.1)	1.208	0.205	5.890	0.71
$(NH_4)_2SO_4$ (0.05)	2.986	0.220	13.570	0.76
NH_4Cl (0.1)	1.208	0.150	6.053	0.50
NH_4Cl (0.05)	1.449	0.110	13.170	0.53
$NH_4H_2PO_4 + NH_4NO_3$ (0.05)	3.820	0.187	20.420	0.92
$(NH_4)_2SO_4 + NH_4H_2PO_4$ (0.05)	3.610	0.193	18.700	0.89
$NH_4H_2PO_4 + NH_4Cl$ (0.05)	3.047	0.176	17.310	0.82

*0.004 M $NH_4H_2PO_4$ + 0.002 M NH_4NO_3

Effect of cultivation temperature: Enzyme production at different incubation temperatures was investigated at an initial media pH: 6.0. The highest I/S ratio (I/S_{max}: 16.92) was measured at 40°C (Table 3). It was reported that the optimal temperature for inulinase production for *Xanthomonas sp.* was 30 to 45°C; for *Pseudomonas sp.*

was 42°C, Park and Yun (2001); for *Penicillium sp.* was 30-33°C, Derycke and Vandamme (1984); for *K. marxianus* was 35°C , Rouwenhorst *et al.* (1990).

Effect of carbon sources: In order to establish the effect of carbon source on production of the enzyme, *R. solani* was grown in media containing various carbon sources and in medium without a main carbon source. The effect of different carbon source on enzyme production is shown in Table 4. The best inulinase production was observed in the presence of inulin (1%) and Jerusalem artichoke powder (3%). Very low activity was observed in media containing soluble starch, pectin and sucrose. This medium's mycelia weights were no detectable. *R. solani* inulinase appeared to be an inducible enzyme. Similar result had been reported for *C. pannarum* inulinase, Xiao *et al.* (1988).

Effect of nitrogen sources: The influence of inorganic nitrogen source on inulinase production was investigated. The inorganic nitrogen source, $NH_4H_2PO_4$ (0.004 M) and NH_4NO_3 (0.002 M), in the production medium (original medium) were replaced by each of $NH_4H_2PO_4$, NH_4NO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ and NH_4Cl at concentrations of 0.05 and 0.1 M. The highest activity was detected in original medium (I/S: 15.34). Very high activity was also observed in media containing $(NH_4)_2HPO_4$ 0.05 M, (I/S: 10.52), (Table 5). Each of nitrogen source in concentration 0.1 M had no stimulant effect. The effects of inorganic nitrogen sources were investigated on inulinase production when they were used in combination. The combined nitrogen source had higher inulinase yields when added individually in the medium (Table 5). It was reported that each fungal species had preference for a specific nitrogen source for optimum production of inulinase, Kochhar *et al.* (1997).

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References

Allais, J.J., S. Kammoun, P. Blank, C. Girard and J.C. Baratti, 1986. Isolation and characterization of bacterial strains with inulinase activity. Appl. Environ. Microbiol., 52: 1086-1090.

Arand, M., A.M. Golubev, J.R.B. Neto, I. Polikarpov, R. Wattiez, O.S. Korneeva, E.V. Eneyskaya, A.A. Kulminskaya, K.A. Shabalin, S.M. Shishliannikov, O.V. Chepurnaya and K.N. Neustroev, 2002. Purification, characterisation, gene cloning and preliminary X-ray data of exo-inulinase from *Aspergillus awamori*, Biochem. J., 362: 131-135.

Azhari, R., A.M. Szlak, E. Ilan, S. Sideman and N. Lotan, 1989. Purification and characterisation of endo-and exo-Inulinase. Biotech. Appl. Biochem., 11: 105-117.

Barthomeuf, C., F. Regerat and H. Pourrat, 1991. Production of inulinase by new mold of *Penicillium rugulosum.* J. Ferment. Bioeng., 72: 491-494.

Derycke, D.G. and E.J. Vandamme, 1984. Production and properties of *Aspergillus niger* Inulinase. J. Chem. Tech. Biotechnol., 34: 45-51.

Gupta, A.K., P. Rathore, N. Kaur and R. Sing, 1990. Production, thermal stability and immobilization of inulinase from *Fusarium oxyporum.* J. Chem. Biotechnol., 47: 245-257.

Haraguchi, K., K. Hayashi and T. Kasumi, 1990. Purification and some properties of endoinulinase from *Chrsosporium pannorum.* J. Ferment. Bioeng., 67: 244-248.

Kochhar, A., N. Kaur and A.K. Gupta, 1997. Inulinase from *Aspergillus versicolor*: A potent enzyme for pruduction fructose from inulin. J. Sci. Ind. Res., 56: 721-726.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Nguyen, Q.D., F. Mattes, A. Hoschke, J. Rezessy-Szabo and M.K. Bhat, 1999. Production, purification and identification of fructooligosaccharides produced by β-fructofuranosidase from *Aspergillus niger* IMI 303386. Biotech. Lett., 21: 183-186.

Ongen-Baysal, G., S. Sukan and N. Vassilev, 1994. Production and properties of inulinase from *Aspergillus niger.* Biotech. Lett., 16: 275-280.

Parekh, S. and A. Margaritis, 1985. Inulinase (β-fructofuranosidase) production by *Kluyveromyces marxianus* in bath culture. Appl. Microbiol. Biotechnol., 22: 446-448.

Park, J.P. and J.W. Yun, 2001. Utilization of chicory roots for microbial endoinulinase production. Lett. Appl. Microbiol., 33: 183-187.

Pessoni, R.A.B., R.L.C. Figueiredo-Ribeiro and M.R. Braga, 1999. Extracellular inulinase from *Penicillium janczewskii,* a fungus isolated from the rhizosphere of *Vernonia herbacea* (Asteraceae). J. Appl. Microbiol., 87: 141-147.

Rouwenhorst, R.J., M. Hensing, J. Verbakel, W.A. Scheffers and J.P. Van Dijken, 1990. Structure and properties of the exratacellular inulinase of *Kluyveromyces marxianus* CBS 6556 . Appl. Environ. Microbiol., 56: 3337-3345.

Wei, W., S. Wang, X. Zhu and W. Wan, 1999. Isolation of mutant of *Kluyveromyces sp.* Y-85 resistant to catabolite repression. J. Biosci. Bioeng., 87: 816-818.

Xiao, R., M. Tanida and S. Takao, 1988. Inulinase from *Chrysosporium pannorum.* J. Ferment. Technol., 66: 553-558.