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Invertase activity of psychrotrophic fungi

Aktywność inwertazowa grzybów psychrotroficznych

SUMMARY

A total of 98 strains of moulds were isolated from arctic tundra soil (Spitzbergen). Among these strains *Cladosporium herbarum* 1, the most effective for production of invertase, was selected by the cup-plate method. The basal medium was optimized in order to improve invertase production by *C. herbarum*. Concentrating (10-fold) of the culture fluid in a vacuum evaporator shows to be the best way both for protein concentration and invertase activity (about 96% of initial activity). Some properties of crude preparation of invertase were examined. Invertase showed its maximum activity at 50°C and pH 6.0, but optimum temperature for enzyme production by *C. herbarum* 1 was at 20°C.

STRESZCZENIE

Z próbek arktycznej gleby (Spitzbergen) wyizolowano 98 szczepów grzybów nitkowatych. Wśród tych szczepów *Cladosporium herbarum* 1 charakteryzował się najwyższą aktywnością inwertazy oznaczanej metodą testu kolumienkowego. Zoptymalizowano skład podłoża hodowlanego dla syntezy tego enzymu przez *C. herbarum* 1. Spośród zastosowanych metod zagęszczania najwyższy odzysk enzymu (około 96% aktywności początkowej) uzyskano poprzez 10-krotną koncentrację w wyparce próżniowej. Określono niektóre właściwości surowego preparatu. Enzym charakteryzował się maksymalną aktywnością w temperaturze 50°C i pH 6.0, jednakże optymalna temperatura wzrostu *C. herbarum* 1 dla syntezy inwertazy wynosiła 20°C.

Key words: invertase, psychrotroph, screening, arctic fungi, *Cladosporium herbarum.*

INTRODUCTION

Inulinases (2,1-β-D-fructan fructanohydrolases, EC 3.2.1.7) hydrolyze inulin (β-2–1-linked fructose polymer with a terminal glucose unit) to fructose and oligosaccharides in a single step and are used in the production of high-fructose syrups (1, 12, 23). Many of these fructosyl-hydrolytic enzymes (invertases and inulinases) possess a wide substrate specificity, the classification of whose type has been dependent on whether the sucrose to inulin hydrolytic ratio (S/I ratio) is higher or less than 1 (17–20).

Invertase (β-D-fructofuranoside fructohydrolase, E.C.3.2.1.26) catalyzes the cleavage of sucrose to glucose and fructose. Invertase is one of the most widely used enzymes in food industry, especially in the preparation of jams and candies (14, 23). It has been extensively studied in yeast and *Neurospora* sp. The enzyme is a glycoprotein, with mannose being the major component of the carbohydrate moiety (3, 4, 8, 9).

Despite their numerous potentials of application in fundamental and applied fields, microorganisms isolated from cold environments have received little attention especially in comparison to thermophiles. Their advantages and both their ecological and economical values may not yet have been realized sufficiently. Nevertheless, in the past decade an increasing interest of biotechnologists in these extreme organisms could be observed (2, 10). Cold-adapted (psychrophilic and psychrotrophic) microorganisms are distinguished from mesophiles by their ability to grow at low temperatures. Under such condition apart from other physiological characteristics — psychrophiles and psychrotrophs have slower metabolic rates and higher catalytic efficiencies than mesophiles. Cold-adapted microorganisms have a considerable potential in biotechnological application such as waste treatment at ambient temperatures, enzymology, food industry and medical applications (10, 11). The application of cold-adapted microorganisms offers numerous advantages: high microbial growth rates as well as high enzymatic activities and catalytic efficiencies in the temperature range of 0–20°C prevent the risk of microbial contamination (especially in continuous systems), may shorten process times and allow to renounce on expensive heating/cooling systems thus constituting a considerable progress towards the saving of energy.

There have appeared a number of reports concerning hydrolytic enzymes production by psychrophilic microorganisms (2, 10, 11); however to our knowledge, no investigations have been performed on invertase.

As the first step toward characterization of cold-adapted enzymes we report the invertase activities of arctic fungal isolates.

MATERIALS AND METHODS

Microorganisms and media

Fungi were isolated from soils in the Calypsostrandy situated in southern Bellsund region (lat 77°33′ N long 14°30′ E), Wedel Jarlsberg Land (West Spitsbergen). Composition of Martin medium used for screening of microorganisms was as follows: glucose, 1%; peptone, 0.5%; K_2HPO_4, 0.1%; $MgSO_4 \times 7H_2O$, 0.05%; rose bangal (sterilized separately) 0.003%. After sterilization 0.003% of streptomycin was added.

The composition of basal medium (BM) used for catalase production was as follows: inulin, 2.0%; yeast extract, 0.5%; $NaNO_3$, 0.2%; $MgSO_4 \times 7H_2O$, 0.01%; KH_2PO_4, 0.05%.

Taxonomic characterization of fungi

Identification of isolated moulds strains was achieved on the basis of their micro- and macromorphological features, expressed in microcultures, and on agar plates containing known diagnostic media for moulds, by the method of Domsch et al. (6) and Onions and Brady (16).

Isolation of invertase producing microorganisms

Isolation was completed by means of a dilution plate method. Portions of 1.0 g of representative soil were placed in a dilution tube containing 9 ml of sterile distilled water, and shaken for 15 min. Serial 10-fold dilutions were made and surface inoculated onto agar plates of Martin medium plates were incubated for 8–10 days at 15 and 30°C. Fungi were isolated from plates that contained no more than 50 well separated colonies.

Selection of strains for invertase activity

The first selection of invertase active mutants was done on the basis of the magnitude diffusion zone of the enzyme (mm, in diameter) secreted into agar medium by growing colonies. The agar plates (2%, 20 ml) consisted of (g/l): sucrose, 10; starch, 10; citric acid, 1.0; KI, 1.0; $MgSO_4$, 0.5; $NaNO_3$, 2.0; KH_2PO_4, 2.0; sodium deoxycholate, 0.2; and glucose oxidase (100,000 U/mg *A. niger,* Sigma), 0.1 in McIlvaine buffer pH 5.0. The enzyme was added to the hot agar medium at the moment its temperature lowered from 100 to 55°C, but was still a liquid (to prevent heat inactivation of the glucose oxidase). The violet-blue enzymatic zones were formed by iodine released from KI by glucose oxidase and combined with starch (plate method *a*). Such enzymatic zones could also be formed by culture supernatants. In this approach (plate method *b*), the liquids (0.025 ml) and glucose oxidase (0.025 ml) were dropped into a well (8 mm diameter) previously cut out in agar medium consisted of elements as above, excepted for mineral salts, yeast extract and sodium deoxycholate, and incubated 5 h at 30°C.

The strains showing the greatest diffusion areas were further and more accurately examined by test-tube cultivation (22 mm diameter, 12 ml medium) on a rotary shaker (220 rev/min). After 72 h incubation at 30°C and centrifugation (5 min at 3000 g) the invertase or inulinase activities were determined in post-culture liquids by plate method *b*.

Terminal selection

The strains selected on the basis of the above described criteria were incubated in 500 ml conical flasks, each containing 100 ml of medium. The media were inoculated with 2 ml spore suspensions (about 2×10^6 spores) and cultured for 4 days as above. Then mycelium was separated by means of filtration through Miracloth quick filtration material (Chicopee Mills, Inc., New York, USA) and invertase or inulinase activities were measured in the filtrate.

Disruption of mycelium by homogenization

Homogenization was performed in glass Universal Lab. Aid (type MPW-309, Poland) homogenizer with a glass pestle operated at about 7000 rev min^{-1}. A ratio of 3 ml 0.1 M McIlvaine buffer, pH 5.0 to 2.0 g wet weight was applied, followed by twenty passes of the pestle. Intracellular catalase was extracted from the homogenized mycelium with 97 ml of this buffer.

Isolation of proteins

The mycelium was separated from the growing medium by filtration through Miracloth quick filtration material (Chicopee Mills, Inc., USA). The enzyme was recovered from clear supernate fluid: a) by concentration in a rotary vacuum evaporator (Unipan 350, Poland) at 35°C and 50°C; b) ultrafiltration using a TCF-10 cell and Pellicon XL PLCGC membrane (Millipore); c) lyophilization in lyophilizer (Labconco, USA).

Analytical procedure

The extra- and intracellular (measured as cell-free extracts) invertase activity was determined by the 3,5-dinitrosalicylic acid (DNS) method (15). The reaction mixture containing 1% (w/v) sucrose in 0.1M Mc Ilvaine'a buffer (pH 6.0) and enzymes solution were incubated at 50°C for 30 min and the absorbance measured at 550 nm. The amount of reducing sugar liberated was measured using fructose as a standard. One unit (U) of enzyme activity was defined as the amount of the enzyme which produces 1 μmole of reducing sugar per min under these assay conditions. The protein content in the medium and post-culture liquid was determined by the method of Schacterle and Pollack (22). After cultivation, the mycelium dry weight was determined by washing and drying it at 105°C. Fermentations were performed in 3 replicate culture flasks, and analyses carried out in duplicate. The data given here are the means of the measurements. The mean standard error of the enzyme estimate was ± 0.023 and ranged from ± 0.003 to ± 0.032 U/ml.

pH and temperature assays

The influence of pH and temperature on enzyme activity, as well as its thermostability, were investigated in 0.1 M McIlvaine buffer. Thermostability was assayed by 1-h exposure of the enzyme preparation at various temperatures.

RESULTS AND DISCUSSION

A total of 98 strains of moulds were isolated from tundra soils and examined for extracellular invertase activity by the plate method *a*. Among these strains, twenty-six showed extracellular invertase activity (26.6%). Ten strains showing the highest invertase activity were used for further experiments. These included genera *Cladosporium, Mortierella, Penicillium and Aspergillus* as judged from microscopic observation. All these isolates grew well at 4, 20°C and two of them at 30°C, indicating that they were psychrotrophic. These cultures were evaluated for their invertase activity by plate method *b*. The results of these experiments are summarized in Table 1, showing that the best invertase producer was strain *Cladosporium herbarum*. Since the isolates were obtained from representative samples in the environment, we can presume that invertase producing microorganisms are well distributed in arctic tundra soil.

In further studies, the medium was optimized in order to improve secretion of intracellular invertase by *C. herbarum.* Relationship between the concentration

Table 1. Preliminary evaluation of invertase activity of moulds grown on basal medium. Relative activity was expressed in mm of enzymatic zone size formed on agar plates (plate method *b*)

Organism	Number of strains tested	Invertase activity (mm)
Aspergillus versicolor	1	11
Cladosporium cladosporoides	3	8–13
Cladosporium herbarum	1	20
Mortierella minutissima	3	11–15
Penicillium chrysogenum	1	12
Sclerotium sp.	1	12

of glucose or fructose and amount of invertase produced was shown in Table 2. The highest extra- and intracellular invertase occurred in the medium containing inulin (1.5%) and fructose (0.5%) or inulin alone (2%), which is in agreement with earlier studies on invertase from genus *Aspergillus* and *Saccharomyces* (7, 13). However, in this conditions biomass concentration attained its lower value, caused by high pH of the medium. The lowest intracellular invertase activity was reached in the medium containing glucose or glucose and fructose. It may suggest repressive effect of glucose and fructose on invertase production (4).

Table 2. Extracellular invertase activity produced by *Cladosporium herbarum* grown in the basal medium containing different concentrations of glucose and fructose

Component concentration	Invertase activity (U/ml)		Dry (g/l)	Final pH
	Extracelluar	Intracellular		
Sucrose 2%	0	0.17	15.0	6.9
Glucose 2%	0	0.13	4.0	7.1
Inulin 2%	0.15	0.21	3.5	8.2
Fructose 2%	0	0.09	13.0	7.2
Sucrose/glucose 1.5%/0.5%	0	0.17	14.5	7.1
Sucrose/fructose 1.5%/0.5%	0	0.19	13.5	7.2
Glucose/fructose 1.5%/0.5%	0	0.09	10.0	7.4
Inulin/fructose 1.5%/0.5%	0.16	0.29	6.0	7.8
Inulin/glucose 1.5%/0.5%	0	0.25	6.5	7.3

As far as we know, reports on invertase activity of psychrotrophic fungi have not yet been published. Therefore, our results in this respect can be compared with the data concerning mesophilic fungi. The activities of extra- and intracellular invertase produced by *C. herbarum* 1 were significantly lower in comparison with that reported for mesophiles, such as *Aspergillus niger* (21) or *A. ficuum* (8).

Bradner et al. (2) found that antarctic isolates of microfungi had showed

general growth characteristics and hydrolase production resembling those of mesophilic species. However, some strains and enzymes showed adaptation to elevated and/or colder temperatures indicating the development of means to deal with environmental stress generated by non-mesophilic growth temperatures. A relatively good growth and chitinase activity was displayed by *Penicillium* and *Trichoderma* isolates at 10°C as well as protease activity in *Trichoderma* sp. (2).

In subsequent experiments the influence of pH and temperature on enzyme activity, as well as thermostability were investigated. The optimum pH for the activity of invertase secreted into the medium by *C. herbarum* 1 was at the level of 6.0 (Fig. 1). Maximal activity was achieved at temperatures, ca 50°C (Fig. 2). The enzyme is relatively heat and pH resistant. Its activity decreased only by 9.3% after 60 min heating at 50°C. Enzyme was inactivated after 60 min at 70°C or at pH 9.0 (Fig. 3, 4). In literature reports, the data indicate that invertase

Fig. 1. Effect of pH on extracellular invertase activity produced by *Cladosporium herbarum*

Fig. 3. Temperature stability of extracellular invertase produced by *Cladosporium herbarum*

Fig. 2. Effect of temperature on extracellular invertase activity produced by *Cladosporium herbarum*

Fig. 4. Effect of pH on the stability of extracellular invertase produced by *Cladosporium herbarum*

produced by fungi exhibits its maximal activity at temperatures 30–60°C. The enzyme is relatively heat resistant at 50°C. Higher temperatures cause its rapid inactivation (5, 14, 19, 21, 24).

In the last stage of the experiments different methods of protein isolation and concentration from the culture fluid were examined. Invertase preparations have been obtained by evaporation, lyophilization and ultraphiltration. The results presented in Table 3 show that concentration of the culture fluid in a vacuum evaporator was the best way both for protein concentration and invertase activity (about 96% of initial activity). A similar level of enzyme was obtained in the case when culture fluid was concentrated by ultrafiltration on YM-10 membrane. A little lower level of invertase activity was obtained in the fraction after lyophilization (about 82%). A lower effect to the earlier one was obtained by Ettabili and Baratti (8) or Rubio and Maldonado (21), testing invertase from *A. ficuum* and *A. niger.*

Table 3. Influence of various methods of precipitation and concentration of *Cladosporium herbarum* 1 culture fluid on catalase activity

Method	Protein (mg/ml)	Total protein (mg/l)	Invertase activity		
			Total activity U	Activity yield %	Specific activity (U/mg protein)
Culture supernate	0.24	12.0	19.7	100.0	1.64
Concentrated by ultrafiltration	0.59	29.55	229.0	95.0	7.75
Concentrated (10 times) in a vaccum evaporator at:					
35°C	5.39	107.85	78.75	96.98	0.73
50°C	7.48	149.64	53.00	65.27	0.35
Concentrated by lyophilization	0.44	22.2	16.15	81.97	0.72

The results reported here permit to state that the *Cladosporium herbarum* 1 strain selected in our laboratory constitutes a new source of invertase, and at this stage of studies it represents a low enzyme activity. Further optimization of the medium composition and growth conditions is needed for significantly increased production and secretion of invertase activity by *C. herbarum* 1.

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