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Stable Carbohydrolases of Extremophilic Mycelia Fungi

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ABSTRACT. Enzymatic hydrolysis of cellulose to fermentable glucose is the most important technological process among all possible enzyme technologies. Thermophilic fungi are potential sources of enzymes with the view of scientific and commercial interests. From the collection of microscophic fungi isolated from the ecological niches of Georgia at Durmishidze Institute of Biochemistry and Biotechnology of Agricultural University. Thermophilic micromycetes active producers of stable cellulases were selected. Four endoglucanases were purified to homogeneity from *Sporotrichum pulverulentum* J-3, *Aspergillus wentii* S-6, *Aspergillus versicolor* D-3, *Chaetomium thermophile* P-21 culture medium. Some kinetic, physical and chemical properties of purified endoglucanases (molecular mass, isoelectric point, carbohydrates content, pH, temperature optimums, K_m, K_{cat}, V_{max}, K_i, Henries constant K_p, substrate specificity) were studied.

Key words: *microscophic fungi, endoglucanase, thermopile, hydrolysis, purification*

Synthesis of structural polymers such as, cellulose, hemicelluloses, starch, pectin, etc., and not carbohydrate polymer lignin is characteristic of the great majority of higher plants. Enzymes hydrolyzing or oxidizing these polymers are found in plants [1] but the equilibrium of their hydrolysis or oxidative degradation is so strongly shifted toward their synthesis that hydrolysis becomes a negligible process and should not be taken into consideration. Constitutive components of plant-soil system microorganisms and namely the majority of mycelia fungi genera are characterized by high activities of plant biopolymers degrading enzymes. Enzymatic hydrolysis of cellulose, which is the main component of plant mass (makes up to 60% of all plant mass) to fermentable glucose, is the most important technological process among all possible enzyme technologies [2,3]. Microorganisms such as thermophiles, halophiles, acidophiles, alkaliphiles, etc. often are capable to produce enzymes exceeding in stability of the currently used ones [4-6]. Investigation of extremophiles of different taxonomic groups allows to isolate strains-extremophiles producing well balanced stable molecules of enzymes, having the increased resistance against different critical conditions and so much required in a number of industrial processes [7-9]. Special interest

attracts thermophilic/thermotolerant fungi having potential of growth above 20^{0}C and optimum of growth 40-50^{0}C and very rare at 55-65^{0}C. Fungi thermophiles represent heterogeneous physiological group of various genera in the Phycomycetes, Fungi Imperfecti, Ascomycetes, and Mycelia Sterilia [10]. The aim of present investigation was to select extreme thermophilic strains as new isolates and from existing fungi collection, and to evaluate their cellulose degrading enzymes according to heat stability and salient physical-chemical characteristics.

Materials and Methods

Soils, plants and thermal springs from the most hot places of western (subtropical), eastern (steppe), and southern soil-climatic zones of Georgia were used as sources for isolation of mycelial fungi strains. The procedure of fungi strains isolation was performed from primary plating on 8% agar containing medium. The fungi were cultivated in deep conditions at 35-55^{0}C for ten days. The strains were cultivated on the following nutrient mediums containing (in %): microcrystalline cellulose –1.0; corn extract – 0.05; $NaNO_3$ – 0.36; KH_2PO_4 – 0.2; $MgSO_4$ $7H_2O$ – 0.05. Medium 2, microcrystalline cellulose - 20; corn extract – 0.05; KH_2PO_4 – 0.68; $(NH_4)_2SO_4$ – 0.13; $MgSO_4$ $7H_2O$ – 0.56; $CaCl_2$ – 0.2. The strains were grown in Erlenmeyer flasks with 250 or 750 ml on a shaker 180-200 rapids/min in a 30l fermenter (New Branswick, USA). Viscozimetric activity was determined as a result of enzyme action on soluble Na-CMC according to the method modified by Rodionova et al. [11]. Filter paper activity was determined by the method of Ghose [12], based on cellulase to perform hydrolysis of filter paper oligosaccharides, the amount of reducing sugars were estimated according to Adney and Baker [13]. For the determination of cellobiohydrolase activity the method offered by Nummi, et al, was used [14] with amorphous cellulose as a substrate. Toxicity of strains was determined according to [15].

Results and Discussion

Mycological studies exposed the most frequently met genera in various substrates of Georgia: *Mucor, Rhizopus, Chaetomium, Allescheria, Malbranchea, Botrytus, Monilia, Aspergillus, Penicillium, Sporotrichum, Trichoderma, Trichotecium, Alternaria, Cladosporium, Helmintosporium, Fusarium* and the order Mycelia Sterilia. Systematic analysis exposed the existence in collection strains of Ascomycetes, Basidiomycetes, Zygomycetes, Deuteriomycetes, Mycelia Sterilia. Among the collection strains it was found the existence of toxic strains (up to 20%). In the majority of cases they produce different set of cellulases some strains actively form such extracellular enzymes as: xylanase, laccase, Mn peroxidase, alfa- and glucoamylases, acid and neutral proteases, pectinases, invertase, alfa-galactosidase, etc. Tests for thermophilicity, performed by determining of growing potential of strains between 30-60 ^{0}C, showed that 6% of all strains could be considered as thermophiles/thermotolerants. The representatives of the following genera: *Mucor, Aspergillus, Chaetomium, Allesceria, Malbranchea, Sporotrichum* display this property more frequently. Finally, the collection of fungi-thermophiles accounting 36 strains, actively growing between 40-45 ^{0}C on glucose, glycerol and xylose has been created. Most thermophilic strains, in particular, *Allecheria terrestris* I-5 and *Chaetomium thermophile* P-21 grow well at 42-45^{0}C , *Sporotrichum pulverulentim* J-3 at 40^{0}C, *A. versicolor* and *A. wentii* S-6 at 42 ^{0}C. The cellulase system in fungi cultures comprise three cellulases: enzymes endo-(1,4)-β-D-glucanase (endoglucanase), exo-1,4-β-D-glucanase (cellobiohydrolase), β- glucosidase. It should be underlined that not all thermopiles are characterized by production of full set of cellulases. As it was determined above, 90% of all cellulase activities were extracellular, so further only extracellular activities will be discussed concerning the cellulase production potential of strains. The activities of cellulases of cultural

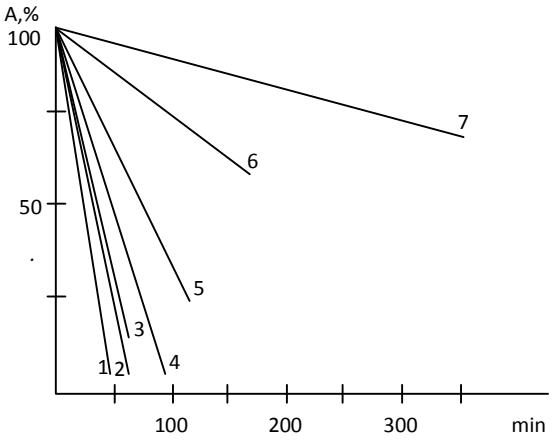

Fig.1. Curves of thermal inactivation of cellulase preparations from thermophilic and mezophile strains, assayed according to filter paper activity at 65^{0}C (Schematized for clarity).
1 - "Onozuka R-10" *(Trichoderma reesei);*
2 - *Spootrichum pulverulentum* J-3*;* 3 - *Aspergillus terreus* K-60*;* 4 - *Aspergillus wentii* S-6*;* 5 - *Aspergillus versicolor* D-3*;* 6-*Chaetomium thermophile* P-21*;*
7 - *Allesheria terrestris* I-5.

filtrates are shown in Table 1. Some specificities of fungi-thermophiles in production of cellulases was clearly expressed while their deep cultivation. For instance, the absence even in traces amount of β-glucosidase in cultural filtrate of *Sporotrichum pulverulentum* J-3 strain should be considered as anomalous. The complete absence or very low activity of extracellular cellobiohydrolase, activity in cultural filtrates of thermophiles indicate on different mechanisms of cellulose enzymatic break down performed by extracellular preparations isolated from thermophiles but it should not be considered as a common characteristic of all fungi thermophiles. Estimation of thermo-stability of selected cellulases was done on further stage via the kinetic curve of thermo-inactivation. Incubation of enzyme solution was carried out without a substrate at 65^{o}C within 300 minutes in order to study the kinetics of thermo-inactivation. Enzyme activity was estimated by the standard methods at particular intervals of time and was calculated by the formula $A/A_0 \cdot 100\%$, where A_0 is an initial activity, and A – residual activity in t-period of time. Kinetics of thermo-inactivation of one and the same enzyme was compared in thermophils and mesophylls, in particular, with the enzyme of *Trichoderma reesei* commercial preparation – Onozuka R. Kinetics of thermo-inactivation of enzyme preparations according to filter paper activity is shown on (Fig.1). The same results were obtained while analyzing heat inactivation of cellulases according to CMC activity. Generally, it should be considered that cellulases from thermophilic fungi are more heat-stable enzymes than their mesophilic analogues. At the same time cellulase of Georgian thermophilic strains attract interest due to their unusual properties. For instance, as it has been mentioned, cellulase preparation from *S. pulvrulentum* J-3 does not contain cellobiohydrolase and β-glucosidase activities but as a result of 40 minutes incubation (exhausted hydrolyses) on crystalline cellulose in reaction mixture is liberated the detectable amount of glucose. With the aim to compare the endoglucanases from thermophilic fungi endoglucanases from different genera were purified and some of their molecular characteristics determined.

Purification of Endoglucanases

Purification of endo-1,4-β-D-glucanases from thermophilic fungi has been performed according to special methodology worked out for this group of enzymes. The common scheme of enzymes purification consists of the following stages: ion exchange chromatography on DEAE Toyepearl, CM-650 Toyepearl, gel-filtration (HW-55), rechromatography on DEAE-Toyepearl. Using this methodology endo-1,4-β-D-glucanases from the following thermophilic fungi strains have been purified to homogenous state: *A. versicolor* D-3 (Degree of Purification 56), *A. wentii* S-6 (D.P.93), *S. pulverulentum* J-3 (D.P.82), *Chaetomium thermophile* P-21 (D.P.46) (Table 2). As a result of the above shown purification scheme, endo-1,4-β-D-glucanase produced by *A. versicolor* D-3 was received in a highly purified state and divided into two fractions, the main one was equal to 43% of initial activity and the minor one to 13% of initial activity. Finally the main fraction exposes protein homogeneity by disk electrophoresis. The existence of homogenous endo-1,4-β-D-

Table 1. Extracellular activities of fungi-thermophiles while their deep cultivation

#	Strain	Activity un/ml			
		endoglucanase	CMC	FP	βglucosidase
1	*A. versicolor*	23.5	19.7	0.8	0.59
2	*A. wentii*	18.5	15.3	0.68	0.5
3	*A. terreus*	4.1	11.5	0.5	0.5
4	*Sp. Pulverulentum*	10	8.7	0.3	_
5	*Ch. Termopile*	1.5	1.2	0.1	0.5
6	*Allesheria terrestris*	3.3	1.7	0.2	0,3

glucanases from *A. wentii* S-6, *S. pulverulentum* J-3, and *Chaetomium thermophile* P-21 has also been detected as a result of the same purification methodology. Some physical and chemical characteristics of homogenous endoglucanases were studied.

Adsorption

Formation of strongly bonded cellulase-cellulose complex [ES] is extremely important for successful hydrolyses of insoluble cellulose. Concluding from the postulate: "Better links – better catalysis", high degree of enzyme adsorption on a substrate greatly determines its technologibility [16]. Therefore, the process of adsorption of endoglucanase preparations and homogenous endoglucanase on insoluble substrate – mycrocristalline cellulose was studied by the modified method of Rabinovich [17]. Calculation of the relative content (b) and coefficient of distribution (Kp) of weakly and firmly adsorbed forms on MCC showed that *A.wentii* S-6 preparation contains 33% of weakly adsorbed endoglucanase with coefficient of disstribution Kp (constant Henry) – 0.031 g/l and 67% of firmly adsorbed one with Kp – 0.83 l/g. *Sp. Pulverulentum* J-3 preparation contains 20% of weakly adsorbed endoglucanase with Kp – 0.008 l/g and 80% of firmly adsorbed one with coefficient of disstribution 0.92 l/g. *Chaeomium thermophile* P-21 preparation contains 38% of weakly adsorbed endoglucanase with Kp – 0.006 l/g and 62% of firmly adsorbed one with coefficient of distribution – 0.656 l/g. The preparation of *A. versicolor* D-3 has a very strongly adsorbed form of endoglucanase – 98% with Kp – 1.6 l/g. The study of adsorptive characteristics of homogenous endoglucanases illustrated their homogeneity according to the stability to adsorb on MCC (Table 3). The pH-optimums of endoglucanases were determined by estimating the enzyme activities in a reaction mixture with different pH buffers (2.5-9.0) under standard assay conditions. Estimations of endoglucanase activities at different temperature (40-70^{0}C) were carried under standard assay conditions too. The Michaelis-Menten constant (Km) and maximal reaction velocity (V_{max}) of purified endoglucanases were

Table 2. Purification of endoglucanases from deep culture of *A. versicolor* D1

Stages of purification		Total activity units	Total protein mg	Specific activity unit/mg	Degree of purification	Retained activity %
Initial enzyme preparation		2500	550	4.5	1	100
DEAE (Zeta Prep) disk		2150	90	23.8	5.3	86
SP (Zeta Prep) disk		1950	70	28	6.2	78
Gel filtration HW-55	E_1	350	8	43.7	9.7	I-14
	E_2	1090	10	109	24.2	II-43.6
DEAE- 650 Toypearl	E_1	325	5.5	59	13.1	I-13
	E_2	450	1.8	250	56	II-18

Table 3. Adsorptive characteristics of crude and homogenous endoglucanases on CMC

Strain	Kp L/g	%	Kp l/g	%
	Firmly adsorbed form		Weakly adsorbed form	
A.wentii	0.83	67	0.031	33
Sp.pulverulentum	0.92	80	0.008	20
Ch. thermophile	0.656	62	0.006	38
A. versicolor	1.6	98		
Homogenous				
A. wentii	1.8			
Sp.pulverulentum	1.6			
Ch. thermophile	1.9			
A. versicolor	2.2			

determined by measuring the enzyme activity with CMC in concentrations ranging from 1 to 10 mg/ml. From the double-reciprocal plots, Kinetic constants were calculated according to Lineweaver-Burk plot. The molecular mass of purified endoglucanases was estimated by 12% sodium dodecyl sulfate polyacrylamide gel electoctrophoresis as described in [18]. High and low molecular weight standards (Sigma) were used to determine molecular weight of enzymes. The gel was stained by silver staining method. A plot of log molecular weight of the standard protein markers against relative mobility gives the molecular weight of protein. Analytical thin-layer gel isoelectric focusing was performed in the pH range of 3.5-9.5. After electro focusing, the gel was fixed in solution containing a methanol/acetic acid solution. Later, it was silver-stained. The pI of endoglucanase was determined using the plot of relative mobility of standard protein markers versus their pI. Inhibition of endoglucanase by glucose was determined in presence of dyed cellulose "DC-31" as substrate in various quantities (2.5-12.5g/l). Inhibition constants (K_i) were determined from corresponding Lineweaver-Burk plots using standard linear regression techniques [19]. All the studied endoglucanases were weakly, non-competitive inhibited by reaction products. The carbohydrate content of purified endoglucanases was determined by the phenol-sulphuric acid method [20]. Some Physical-chemical characteristics of homogenous endoglucanases are given in Table 4. As seen from the data of Table 4, the molecular masses of endoglucanases from different genera of thermophilic fungi are between 45-55 kDa and correspond to literature data for endoglucanases of thermophilic fungi having masses from 30 to 100kDa [21.22]). The great similarity in isoelectric points (pI) has been exposed for all four enzymes (4.5-4.7), which also correlate with the published data [23]. Temperature optimum of enzymes action depending on the prolongation of time of enzyme action is always discussible. In our experiments the hydrolysis of filter paper was continued for one hour, for endoglucanase from *A. versicolor* D-3 and *A. wentii* S-6 temperature optimum of action was equal to 60^0C, for the strain *Chaetomium termophile* P-21 in the same conditions 65^0C, and only the endoglucanase of *Sporotrichum pulverulentum* J-3 has optimum of enzyme action below 60^0C. There is one publication in literature indicating highest temperature optimum of endoglucanase from thermophilic fungi *Talaromyces emersonii* lying between 75-80^0C

[24], in others the temperature optimum of action does not exceed 65^{0}C [9,25]). The content of carbohydrates for the heat stability of fungal endoglucanases might be quite important for the stabilization of molecules. First of all, even slightly heat stable endoglucanases contain carbohydrates in different quantity [26] and the content of carbohydrates in the most stable endoglucanases is equal to 30, 45 and 50% [4]. Content of carbohydrates in endoglucanases of strains under experiment was quite similar 18-22%, with the exception of endoclucanase of *Sporotrichum pulverulentum* J-3 equal 6,7. pH optimum of action for the great majorities of fungal endoglucanases (for both, mesophiles and thermophiles) lays in slightly acidic area (4.0- 5.8).

Substrate specificity

The action of highly purified endoglucanases of thermophilic fungi on different compounds having α and β- bonds: CMC, amorphous cellulose, lichenin, galactane, arabinan, galactomannan, avicel, laminarine, mannan, cellobiose, para-nitrophenyl galactopyranoside was investigated. Concentration of substrates in reaction mixture was from 1.5 up to 7 g/l. Activities of the enzymes were detected according to liberated (formed) reducible sugars. The time for the enzymatic hydrolysis reaction was from 10 minutes to 72 hours. According to the obtained data all enzymes are characterized by great similarity of action on different bonds in substrates. Endoglucanases of thermophilic fungi easily hydrolyze β-1.4 bonds in carboxymetylcellulose. Their action is detectable (measurable) on the following substrates: amorphous cellulose (β-1.4- bonds), lichenin (β-1.4- and β-1.3), galactane (β-1.4-) and mannan (β-1.4-), expose trace activities while action on galactomannan (β-1.4 and α-1.6), laminarin (β-1.3), mannan (β-1.3) and do not act on arabinan (α-1.5), cellobiose, para-nitrophenyl-galactopyranoside, as well as on para-nitrophenyl-glucopyranoside. So it could be suggested that endoglucanases of fungi-thermophiles actively cleave β-1.4- bonds in polysaccharides, have trace activities on β-1.3-bonds, and do not act on other β- or any α-bonds in small or high molecular substrates.

Hydrolysis

It was stated that at the action of homogenous endoglucanases of *A.wentii* S-6, *A.versicolor* D-3, *S.pulverulentum* J-3, *Ch. thermophile* P-21 on nonsoluble MCC substrate for 72 hours (temperature of incubation - 55°C, [MCC] -5g/l, 0.05 M acetate buffer pH 4.5) the degree of conversion is 68%, 75%, 48%, 27%, respectively, and that on amorphous cellulose for 45 hours (55°, [MCC]- 2g/l, 0.05 M acetate buffer pH 4.5) -82%, 87%, 75% and 52%, respectively. Chromatodraphic (HPlC "Waters") analysis of crystalline substrate hydrolysis products by homogenous endoglucanases showed that hydrolysis products contain glucose, cellobiose, cellotetraose. According to the results of hydrolysis (48 hours, [S]-1g/l0ml) of different cellulose containing agrarian wastes (tea, tobacco, wine, beet, citrus) by endoglucanase preparations yield of reducible sugar was 24-58%, yield of glucose 16-30%.

ბიოტექნოლოგია

ექსტრემოფილური მიცელური სოკოების სტაბილური კარბოჰიდროლაზები

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** საქართველოს აგრარული უნივერსიტეტის ს.დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტი, თბილისი

ცელულოზის ფერმენტული ჰიდროლიზი მადუღარ შაქრებამდე უმნიშვნელოვანესია ფერმენტულ ტექნოლოგიებს შორის. თერმოფილური მიკრომიცეტები მეცნიერული და კომერციული თვალსაზრისით პერსპექტიული ფერმენტების წყაროს წარმოადგენენ. აგრარული უნივერსიტეტის ს. დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტის მიკროსკოპული სოკოების კოლექციიდან შერჩეულია აქტიური და სტაბილური ცელულაზების პროდუცენტის თერმოფილური შტამები. შემუშავებულია ოთხი შტამის *Sporotrichum pulverulentum* D-1, *Aspergillus wentii* S-6; *Aspergillus versicolor* D-3; *Chaetomium thermophile* P-21 ცელულაზური კომპლექსის ერთ-ერთი ფერმენტის ენდო-1,4-β-გლუკანაზას გაწმენდის სქემა. დადგენილია ჰომოგენური ენდოგლუკანაზების ზოგიერთი კინეტიკური და ფიზიკო-ქიმიური მახასიათებელი, როგორიცაა მაგ. მოლეკულური წონა, იზოელექტრული წერტილი, ნახშირწყლების შემცველობა, ტემპერატურული და pH ოპტიმუმი, K_m, K_{cat}, V_{max}, K_i, ჰენრის კონსტანტა K_p, სუბსტრატული სპეციფიურობა.

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