საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 3, №1, 2009
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 3, no. 1, 2009

Biochemistry

Selection of Microscopic Fungi - Pectinase Producers

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ABSTRACT. Applying screening, the strains active producers of pectinase have been selected among the microscopic fungi collection available at the Durmishidze Institute of Biochemistry and Biotechnology under conditions of deep cultivation. The most active producers were selected by screening among the cultures of thermophilic, alkali, acidophilic and halophilic microscopic fungi strains.

Active producers have also been taken from ordinary growth conditions of microscopic fungi cultures. The cultures have been selected from the following genera *Aspergillus, Penicillium, Mucor, Trichoderma, Rhizopus, Sporotrichum, Chaetomium* and *Stempillium*. In the collection among pectinase producers representatives of the following genera dominate: *Aspergillus* and *Penicillium*. Among active producers of pectinase note should be taken of - *Penicillium canescens* I-85 (acidotolerant) *Aspergillus niger* T 1-1 (thermotolerant), *Trichoderma viride* Ts-2 (alkalitolerant), which against the background of high pectinase activity were characterized by cellulase and xylanase activities as well.

Myco-toxicological studies have shown that the selected strains are neither toxic nor pathogenic.

The physiology and some biochemical characteristics of the selected strains have been investigated, the nutrient media for each particular strain was optimized and conditions of growth were established.

The strain *Penicillium canescens* I-85 reveals the highest pectinase activity at 27°C and pH 4.0; the strain *Aspergillus niger* T 1-1 at 40°C, pH 6,0; *Trichoderma viride* Ts-2 at 30°C, pH 7.5. As a result of optimization of the nutrient media the activity of pectinase increased by 122, 28 and 98%, respectively. *© 2009 Bull. Georg. Natl. Acad. Sci.*

***Key words**: pectinase, xylanase, extreme halophile, thermotolerant, alkalitolerant, microscopic fungi.*

Introduction.

Microorganisms are considered to be prospective enzyme producing sources [1, 2]. They have a number of advantages: through the application of selection methods increase of biosynthesis via the conditions of cultivation, in-depth interaction on various substrates, wide spectrum of enzyme complex and their application in gene engineering via gene cloning. Mycelial fungi are distinguished for such ability, as they are eukaryotic organisms in comparison with prokaryotes, have wide spectrum of genetic information, and are able to perform microbial conversions.

At present, about 30 enzymes out of 2000 well-known ones are actively used in production. Technological processes, in which enzyme systems of microscopic fungi are involved, are of increasing importance in food industry. The bulk of enzymes on the world market represent hydrolases, particularly pectinases, which participate in many physiological processes occurring in plants: mainly in fruit ripening. They are considered promising in obtaining carbon-containing

compounds possessing immune-modulating activity and are successfully applied in improving the yield and quality of fruit juices [3, 4]. By searching for new effective producers and establishing their optimum cultivation conditions it is possible to obtain enzyme preparations with high pectinase activity.

The selection of microscopic fungi was carried out from strains isolated from different ecological niches of the South Caucasus.

The action of temperature, pH and NaCl in different concentrations on the growth and development of strains has been investigated with a view to exploring the extent of their extremophilicity .

The microscopic fungi were cultivated in the temperature range of 5-55ºC, with the temperature interval of 5ºC, and at pH in the range from 2.0 to 10.0 with the interval value of 0.5 in the starting nutrient medium.

We have taken as the temperature and pH optimums the microscopic fungi which have provided the maximal colony growth, detected according to the measurements of colony diameter and to the growth rate.

For galophilic cultures discrimination, we have added NaCI to the starting nutrient medium in different concentrations from 0.5 M to 4 M (respectively 2.93% - 23.2%).

The screening of enzyme producers was conducted in submerged cultivation. 10-day conidia culture suspension served as the cultivation materal.

Deep cultivation was carried out in 750 ml Erlenmeyer flasks, on temperature-controlled rotary shaker (180-200 rpm), at 30-45°C. In order to reveal cellulase, xylanase and pectinase producers, cultivation was conducted during 90-96, 70-76 and 96-108 hours, respectively. The amount of nutrient media equaled 100 ml.

The media was applied as liquid nutrient media.

Composition of liquid medium for pectinase production: ***Medium #1,*** *in %:*

Apple pectin –1.0; KH_2PO_4 – 0.3; $(NH_4)_2$ SO_4 – 0.25; $MgSO_4$ – 0.3; (pH 4.5-5.0).

Composition of liquid medium for extra cellular cellulase production: **Medium #2,** in %: microcrystalline cellulose – 1.0; $NaNO_3$ – 0.3; KH_2PO_4 – 0.2; $MgSO_4 \times 7H_2O$ – 0.05; corn steep extract - 1.5ml (pH 4.5-5.0).

Composition of liquid medium for extra cellular xylanase production: **Medium #3**, in %: Soy bean flour – 3.0; Na_2HPO_4- 1.5; $(NH_4)_2SO_4$- 0.2; KCl-0.05; $MgSO_4$- 0.015.(pH -4.5).

Pectinase activity was estimated by the method of Witaker, described by Rodionova et al with some modifications [5]

Reducible sugars were determined by the method of DNS[6].

Michael methods for that of xylanase were used [6].

Total cellulase activity was determined by the method of Ghose, based on the potential of cellulase to perform hydrolysis of insoluble substrates (in particular, filter paper) to soluble monosaccharides and oligosaccharides [7]. Reducing sugars were estimated by the method of DNS.

Zoopathogenicity of the studied cultures was investigated by intravenous injection of the fungal suspension in experimental rabbits [8].

The method of Berestetsky [9] was used to establish phytopathogenicity.

Toxicity was studied by Diekman method [10].

Results and discussion

Since natural conditions play a significant role in the growth, development and physiological functions of microscopic fungi [11,12], cultures were isolated from different soil-climatic zones to obtain diverse experimental material. Microscopic fungi were selected among the cultures isolated from different soil-climatic zones.

As a result of the studies, active producers of pectinase have been found among the various genera of microscopic fungi, characterized by extremophile properties (Table1).

The active producer pectinase strains were selected through screening microscopic fungi cultures under submerged cultivation.

In order to select pectinase producers, the screening was conducted among the cultures of microscopic fungi under conditions of deep cultivation.

It was found that pectinase producers, which produce cellulose and xylanase as well, are mostly representatives of the genera *Aspergillus*, *Trichoderma*, *Sporotrichum* and *Penicillium*. Among them extremophilic cultures (thermophiles, alkali- and acidophiles and halophiles) of microscopic fungi were revealed.

Finally three cultures were selected for further experiments: - *Penicillium canescens* I-85 (acidotolerant) *Aspergillus niger* T 1-1 (thermotolerant), *Trichoderma viride* Ts-2 (alkalitolerant).

Myco-toxicological studies showed that the selected strains are not pathogenic and toxic and may be successfully used for various industrial and agricultural purposes.

The vitality and potential of a microorganism to produce enzymes intensively much depends upon the

Table 1.

Pectinase, xylanase and cellulase activities in cultural filtrates of mesophilic and extremophilic mycelial fungi strains (units/ml)

	Culture	FP	X	P	Characterization
1	*Aspergillus niger* T 1-1	0.56	8,8	12.8	Thermotolerant
2	*Aspergillus niger* G 2-11	trace	6.0	5.5	Moderate halophile
3	*Aspergillus niger* G -1	–	4.5	5.2	Thermotolerant
4	*Aspergillus niger* M 1-2	-	-	4.0	Thermotolerant
5	*Aspergillus niger* M 3-5	trace	trace	8.7	Thermotolerant
6	*Aspergillus niger* S 87	0.44	3.5	4.5	Thermotolerant Alkalitolerant Moderate halophile
7	*Aspergillus niger* H 6-2	-	3.0	6.0	Alkalitolerant
8	*Aspergillus niger* K6-11	-	11.0	4.0	Moderate halophile
9	*Aspergillus niger* N2-2		3.2	2.3	Alkalitolerant
10	*Aspergillus niger* N2-5	0.83	6.0	6.3	Alkalitolerant
11	*Aspergillus* sp. Av 10	0.80	8.0	4.6	Alkalitolerant Thermotolerant Moderate halophile
12	*Aspergillus* sp. Sh 86	0.56	4.0	5.0	Alkalitolerant Thermotolerant Moderate halophile
13	*Chaetomium thermophile* S 26	0.50	5.5	4.5	Thermophile
14	*Chaetomium thermophile* S 48	0.85	10.0	6.0	Thermophile
15	*Penicillium canescens* I-85	0.63	24.0	20.0	Acidotolerant
16	*Penicillium canescens* #2	0.50	trace	17.5	Acidotolerant
17	*Penicillium canescens* AEM 85	0.75	11.0	18.5	Acidotolerant
18	*Penicillium* sp. β 2	0.64	trace	5.2	Alkalitolerant
19	*Penicillium* sp. K 1-7	0.90	-	4.6	Moderate halophile
20	*Penicillium* sp. Sh.60	0.90	-	3.0	Mesophile
21	*Penicillium* sp. K 3-17	-	-	7.0	Extreme halophile
22	*Penicillium* sp. K-5	-	-	3.5	Extreme halophile
23	*Penicillium* sp Tn 1-2	-	4.0	3.0	Mesophile
24	*Sporothrichum pulverulentum* S2-2	0.66	6.5	8.0	Thermophile
25	*Sporothrichum pulverulentum* S 9-2	0.75	7.0	6.0	Thermophile
26	*Trichoderma koningii* B-9	trace	–	23.0	Mesophile
27	*Trichoderma* sp.Ts-2	0.56	5.2	14.9	Alkalitolerant
28	*Trichoderma viride* N2-3	0.90	trace	7.0	Alkaliphile
29	*Trichoderma lignorum* Sh 7-9	1.20	4.0	6.0	Mesophile

FP - Filter paper activity, **X**- Xylanase activity, **P** – Pectinase activity

selection of the appropriate nutrient medium, in particular, carbon, nitrogen, phosphorus sources; yet, carbon source plays a special role in microorganism biosynthesis. It is through repression and induction of a carbon source that regulation of enzyme synthesis takes place [13, 14].

Zosteran, pectines of beetroot, citrus, apple and whey in different concentrations, fructose, galactose, lactose and glucose – mono- and disaccharides, at the amount of 0. 8%, according to carbon consistence were tested as carbon sources in order to optimize optimum nutrient media for pectinase producers – strains of microscopic fungi. Nutrient medium without carbon source was considered as control.

Introduction of glucose and fructose into the cultivation medium as carbon source was found to cause

inhibition of enzyme synthesis while introduction, of galactose and lactose induced enzyme synthesis in a small amount. Zosteran, pectin from seaweed (family *Zosteraceae*) proved to be an effective inductor.

Induction of the most intensive pectinase activity is caused by application of beetroot pectin and different concentrations of whey as source of carbon. To determine optimal concentration of whey, the whole amount of whey obtained from 1 liter of milk was considered as 100% – the first variant, in the second variant 100 ml of whey was added to 900 ml of water (10% of previous variant) and finally, 10 ml of whey was added to 990 ml of water (1% of the first variant). Almost all strains revealed high pectinase activity in the medium, to which 10% of whey was introduced. The influence of beetroot pectin and different concentrations of whey on pectinase activity is given in Table 2, by examples of three different genera.

As is seen from the Table, all three strains display high activity in the presence of 10% whey. However, they differ slightly in relation to beetroot pectin; particularly, a representative of *Trichoderma* is characterized by relatively high activity in the medium containing beetroot pectin.

As mineral sources of nitrogen, the following salts were applied: ammonium nitrate, ammonium chloride and ammonium sulfate. Among organic sources of nitrogen, peptone and casein were used. Nutrient medium without a source of nitrogen was considered as control. Finally, it was found that peptone, casein and ammonium sulfate – $(NH_4)_2SO_4$ were utilized well by almost all selected strains. Most strains displayed maximum activity upon application of ammonium sulfate. It was found that a correct option of ammonium sulfate concentration is important as well. In nutrient media, ammonium sulfate was introduced at the amount of 0.25–5.0 g/l (Table 3).

In order to select the source of phosphorus, the most widely used compounds for nutrient medium – monosubstituted sodium phosphate, disubstituted ammonium phosphate and mono- and disubstituted potassium phosphate were tested. The introduction of monosubstituted potassium phosphate into the medium was optimal for the production of pectinases.

In order to obtain pectinase activity the following media were considered as optimum for almost all strains:

Composition of liquid medium for pectinase production: ***Medium #1,*** *in %:*

Beetroot pectin –1.0; KH_2PO_4 – 0.3; $(NH_4)_2SO_4$ – 0.25; $MgSO_4$ – 0.3; (pH 4.5-5.0).

Composition of liquid medium for pectinase production: ***Medium #1*,*** *in %:*

Table 2

Activities of pectinase in cultural filtrates of mycelial fungi strain at different source of carbon, (units/ml)

Source of carbon	*Aspergillus niger* T 1-1	*Penicillium canescens* I-85	*Trichoderma* sp.Ts-2
Beetroot pectin	10.3	17.4	14.5
Whey,100%	11.4	13.8	10.8
Whey, 10%	12.2	19.5	12.4
Whey, 1%	9.5	8.9	8.3
Control	1.2	0.9	0.7

Table 3

Activities of pectinase in cultural filtrates of mycelial fungi strains at different concentrations of ammonium sulphate, (units/ml)

$(NH_4)_2SO_4$ g/l	*Aspergillus niger* T 1-1	*Penicillium canescens* I-85	*Trichoderma* sp.Ts-2
0.25	6.8	9.4	7.5
0.5	7.5	14.2	8.3
0.75	8.3	17.0	10.5
1.0	9.4	18.3	11.9
2.5	12.0	20.0	13.5
5.0	11.2	19.2	13.4
Control	7.5	2.4	2.7

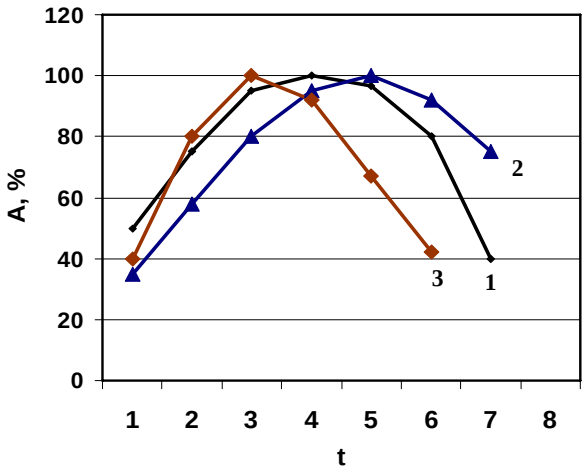

Fig. 1. Effect of temperature on the action of pectinase.
Strains - 1.Trichoderma viride Ts-2; 2. Aspergillus niger T1-1; 3. Penicillium canescens I-85.

KH_2PO_4 – 0.3; $(NH_4)_2$ SO_4 – 0.25; $MgSO_4$0.3; 10% (100 ml) – whey; pH 4.5-5.0).

The results of studies of the effect of carbon sources have shown that xylanase from selected cultures is the induced enzyme.

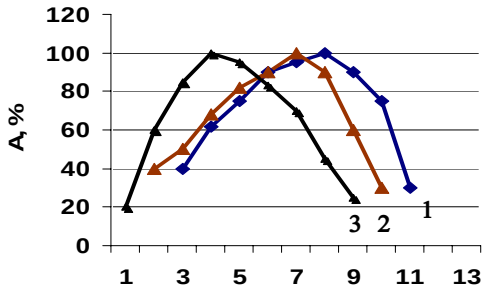

Fig. 2. Effect of pH on the action of pectinase.
Strains - 1.Trichoderma viride Ts-2; 2. Aspergillus niger T1-1; 3. Penicillium canescens I-85.

T1-1 – at 40°C, and *Trichoderma viride* Ts-2– at 30°C (Fig.1).

The optimal pH for the cultivation of these strains was also determined. Since the strain *Trichoderma viride* Ts-2 was alkalitolerant, submerged cultivation was carried out in ranges of pH from 6.5 to10.0.

The highest amount of pectinase is produced at pH 7.5, in more alkaline medium the activity is reduced, while at pH 6.0 only 25% of the activity is retained. In case of growing at neutral pH the activity of extracellular pectinase equals 0 (Fig.2).

Table 4.

Microscopic fungi strains active producers of pectinase

№	Strain	Initial atividies, unit/ml	Activities unit/ml, after optimization of nutrient media	Activities unit/ml, after estimation in optimal conditions	%, of increased activities
1	*Penicillium canescens* I-85	9.0	20.0	20,0	122
2	*Aspergillus niger* T 1-1	10.0	12,0	12.8	28
3	*Trichoderma viride* Ts-2	7.5	13.5	14.9	98

In much as temperature is extremely important in microorganism growth regulation and physiological activity, the effect of temperature on the enzyme production was studied in the first place. Taking into consideration the temperature optimum of growth of enzyme active producers, their submerged cultivation was performed within 30°C and 55°C, with 5°C as intervals.

Penicillium canescens I-85 was found to reveal high pectinase activity at 27°C, the culture of *Aspergillus niger* T 1-1 cultures the optimal initial pH was respectively 4.0 and 6.0. Determination of optimum growth conditions, optimization of nutrient media increased the activities of enzymes by 28-122% (Table 4).

Acknowledgements. The designated Project has been fulfilled by financial support of Georgian National Science Foundation, Grant# GNSF/ ST06/ 6-087.

ბიოქიმია

პექტინაზას პროდუცენტი-მიკროსკოპული სოკოების სელექცია

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დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტი, თბილისი

(წარმოდგენილია აკადემიის წევრის გ.კვესიტაძის მიერ)

დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტში არსებული მიკროსკოპული სოკოების კოლექციიდან სკრინინგის გზით, სიღრმული კულტივირების პირობებში შერჩეულია შტამები –პექტინაზის აქტიური პროდუცენტები. პექტინაზის პროდუცენტებს შორის განსაკუთრებით მაღალი აქტივობებით ხასიათდებოდნენ *Aspergillus*-ის და *Penicillium*-ის გვარის წარმომადგენლები. შერჩეული პექტინაზის პროდუცენტებიდან დიდი ინტერესი გამოიწვია სამმა შტამმა*: *Penicillium canescens* I-85 (აციდოტოლერანტი), *Aspergillus niger* T 1-1 (თერმოტოლერანტი), *Trichoderma viride* Ts-2 (ალკალიტოლერანტი), რომლებიც პექტინაზის მაღალი აქტივობის ფონზე ხასიათდებოდნენ ასევე ცელულაზისა და ქსილანაზის აქტივობით. მიკოტოქსიკოლოგიური კვლევებით დადგენილია, რომ შერჩეული შტამები არ არიან პათოგენურები და ტოქსიკურები.

აღნიშნული შტამების ფიზიოლოგიური და ბიოქიმიური თვისებების შესწავლით, სიღრმული კულტივირების პირობებში მოხდა საკვები არეების ოპტიმიზაცია და ოპტიმალური პირობების (ტემპერატურა, pH) დადგენა.

ოპტიმალური პირობების დადგენით, საკვები არეების შემადგენელი კომპონენტების ოპტიმიზაციით შტამების მიერ წარმოქმნილი პექტინაზას აქტივობა გაზრდილია შესაბამისად 122, 28 და 98 %-ით.

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Received December 2008