Selection of Mycelial Fungi Producers of Stable forms of Cellulases, Xylanases and Laccase

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Abstract. Project is focused on obtaining stable, industrially robust enzymes: cellulases, xylanases and laccases for lignocellulosics effective degradation and further fermentation by yeasts to ethanol. Selection of producers of stable cellulases, xylanases and laccase among the diverse mesophilic and thermophilic mycelial fungi strains collection of DIBB, AUG has been conducted. Screening of microscopic fungi strains of the culture collection according to their ability to produce stable and active extracellular enzymes have been carried. Around 400 strains of genera *Aspegillus, Chaetomium, Cladosporium, Fusarium, Helmintosporium, Mucor, Penicillium, Rhizopus, Trichoderma, Trichothecium Myrothecium, Penicillium, Stachybotrys*and*Sporothrichum* were applied in studies. 48 microscopic fungi producer strains have been selected: 19 mesophilic and 29 extremophilic; 15 strains were distinguished by high activities of cellulase/xylanase, 8 strains – by xylanaseand 15 strains by only cellulase production. Screening allowed to select 21 strains of the basidialfungi laccase producers. With the purpose to increase the biosynthesis of the strainsenzymes physiological parameters of cultivation of the mycelial fungi producers have been carried out.

Keywords: mycelial fungi, basidial fungi, cellulases, xylanases, laccase, submerged cultivation

1. Introduction

Although various microorganisms have been evaluated for their ability to deconstruct lignocellulosic biomass, only few have demonstrated production levels compatible for industrial applications (Lynd *et al*., 2002; Demain *et al*., 2005;).The ascomycetes, represented by *Trichoderma ressei*, secrete cellulolytic and xylanolytic enzymes that act in synergy to hydrolyze polysaccharide polymers to glucose, xylose and arabinose, which can be fermented to biofuels. Apart from *Trichoderma*, the other mesophilic strains producing cellulases are *Allesheria terrestris, Chaetomium thermophile,Fusarium oxysporium, Piptoporus betulinus, Penicillium echinulatum, P. purpurogenum, Aspergillus niger, A. wentii, A. versicolor,A. fumigatus* etc. (Kvesitadze *et al*., 1999; Martins *et al*., 2008). In the majority of the cases the cellulases produced by strains of genus *Aspergillus* usually have high β-glucosidase activity and average endoglucanases levels (with some exceptions), whereas strains representing *Trichoderma* have high endo enzyme and low β-glucosidase, and hence has limited efficiency in cellulose hydrolysis. The most cellulases from fungi-mesophiles expose optimum activity at slightly acidic pH (5.0–6.0) and at temperatures between 40 and 55°C. Cellulases used in biotechnology are derived from well-characterized non-extremophilic microorganisms and there is a very little information regarding cellulases from extremophiles. An important drawback of these commonly used industrial enzymes is the lack of activity at even slightly elevated temperature and the tendency of these enzymes to denature at elevated temperatures or other critical conditions. Stable cellulases could be obtained either by isolating extremophilic microorganisms where such unique properties of extremophilic cellulases already exist or by protein engineering (Viikari *et al*., 2007). Thus, thermophillic fungi and bacteria such as *Allesheria terrestris, Chaetomium thermophile, Sporotrichum thermophile, Scytalidium thermophillum, Clostridium straminisolvens, Thermonospora curvata, Pyrococcus furiosus, Acidothermus cellulolyticus*, and *Saccharophagus degradans* producing cellulase complex may be valuable sources of heat stable cellulases (Kvesitadze *et al*., 1999; Kato *et al*., 2004). Systematic studies of these fungi identify some promising candidates for industrial application.

The aim of the project is to obtainstable enzymes from DIBB unique extremophilic mycelial fungi collection for the creation of biotechnology of production of fuel-bioethanol from agricultural and industrial lignocellulosic wastes.

2. Materials and Methods

For the selection of cellulases and xylanases producers, submerged cultivation ofmicroscopic fungi has been carried out in 250-ml Erlenmeyer flasks with 50 ml nutrient medium on temperature controlled rotary incubation shaker (180-200 rpm), at 30-45°C.Composition of liquid medium for production of extracellular cellulases, in %: microcrystalline cellulose – 1.0; $NaNO_3$ – 0.3; KH_2PO_4 – 0.2; $MgSO_4 \cdot 7H_2O$ – 0.05; corn steep extract – 1.5ml (pH 4.5-5.0).

Composition of liquid medium for production of extracellular xylanases, 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).

The strains 10-day old conidial suspensions were used as inocula. Cultivation was carried out during 90-96 hours in case of cellulases and during 70-76 hours, in case of xylanases.

For optimization of enzymessynthesis,cultivation at different pH, temperature and aeration have been conducted. Studied enzymes activities were measured in culture filtrates.

Composition of liquid medium for production of laccases (g per liter): KH_2PO_4 – 0.8; K_2HPO_4–0.3; $MgSO_4$– 0.5; NH_4NO_3 - 2; $CuSO_4$ – 0.25; yeast extract - 3.0, pH 5.8.

Each 250ml erlenmeyer flask contained 50 ml nutrient medium and 5g tangerine peels as a carbon source. In order to reveal laccase producers cultivation was conducted during 6 and 12 days.

Results and discussion

373 strains of DIBB culture collection, representatives of different genera of microscopic fungi: *Aspegillus, Chaetomium, Cladosporium, Fusarium, Helmintosporium, Mucor, Penicillium, Rhizopus, Trichoderma, Trichothecium, Sporothrichum. Chephalosporium*, *Chephalosporium* and *Stachybotrys* were screened on cellulose degradation enzyme activities. According to literary data and our experience these strains are known to be active producers of cellulases and xylanases.

As a result of screening, 48 Strains including 19 mesophiles and 29 extremophiles were revealed as enzyme producers; among them 15 strains were distinguished by high activities of cellulase/xylanase, 8 strains – by xylanase and 15 strains by only cellulase production (Table 1).

Table 1. Cellulase and xylanase activities in cultural filtrates of mesophilic and extremophilic mycelial fungi strains

No	Culture	Cellulase activity, (*FP) U/ml	Xylanase activity, U/ml	Characterization
1	*Aspergillus niger* K 6-11	-	11.0	Moderate halophile
2	*Aspergillus niger* N2-2	-	3.2	Alkalitolerant
3	*Aspergillus niger* N2-5	0.83	6.0	Alkalitolerant
4	*Aspergillus* sp. Av 10	0.80	8.0	Alkalitolerant Thermotolerant Moderate halophile
5	*Aspergillus* sp. Sh 86	0.56	4.0	Alkalitolerant Thermotolerant Moderate halophile
6	*Fusarium* sp.Av.42	0.44	-	Mesophile
7	*Fusarium* sp.Av.61	0.40	-	Mesophile
8	*Fusarium* sp. Sh-33	0.50		Mesophile
9	*Mucor* sp. Sh 81	0.50	-	Thermotolerant
10	*Penicillium* sp. K 1-7	0.90	-	Moderate halophile
11	*Penicillium* sp. Sh.60	0.90	-	Mesophile
12	*Penicillium* sp Tn 1-2	-	4.0	Mesophile
13	*Penicillium* sp.Tn 2-3	0.50	2.0	Mesophile
14	*Penicillium* sp. Av 1	0 34		Alkalitolerant
15	*Sporothrichum pulverulentum* S2-2	0.56	-	Thermophile
16	*Trichoderma viride* N 5-2	0.86	5.2	Alkalitolerant
17	*Trichoderma viride* N2-3	0.90	trace	Alkaliphile
18	*Trichoderma lignorum* Sh 7-9	1.20	4.0	Mesophile
19	*Aspergillus niger* A 7-5	-	20.0	Thermotolerant
20	*Aspergillus* sp. L 4-0	0.56	3.2	Thermotolerant
21	*Aspergillus*sp. V 2-1	0.80	12.0	Mesophile

22	*Aspergillus*sp. J1-3	0.60	9.0	Mesophile
23	*Aspergillusniger* S 87	0.64	6.0	Alkalitolerant
24	*Aspergillus niger*Aj 38	trace	25.0	Thermotolerant
25	*Helmintosporium* sp. I 1-8	0.72	trace	Mesophile
26	*Penicillium canescence* D85	1.05	32.0	Acidotolerant
27	*Sporotrichum pulverulentum* T 5-0	0.84	12.0	Thermophile
28	*Trichoderma viride* X33	0.94	trace	Alkalitolerant
29	*Trichoderma viride* D 13	0.90	trace	Mesophile
30	*Aspergillus niger*V2-4	0.64	15.0	Alkalitolerant
31	*Aspergillusniger* H 6-1	-	25.0	Mesophile
32	*Aspergillus terreus* V2-8	0.70	3.2	Thermotolerant Alkalitolerant
33	*Aspergillus flavus* G 5-1	0.38	22.0	Alkalitolerant
34	*Chephalosporium* sp. O 4-1	-	11.5	Mesophile
35	*Chaetomium*sp.Y 3-1	0.84	9.0	Thermotolerant
36	*Penicillium* sp. Y 1-2	0.80	7.5	Mesophile
37	*Fusarium*sp. Z 7-1	0.58	-	Psychrotolerant
38	*Rhizopus*spV 3-7	0.44	trace	Mesophile
39	*Rhizopus* spV 4-6	0.56	12.0	Alkalitolerant
40	*Aspergillus terreus* S k 2	0.64	15.0	Thermotolerant
41	*Chaetomium* sp. Sy 11	0.84	5.0	Thermophile
42	*Chaetomium* sp. Sk 20	0.70	3.2	Thermotolerant
43	*Myrothecium* sp. Sy 9	0.92	trace	Mesophile
44	*Myrothecium* sp. My 21	0.84	trace	Mesophile
45	*Mucor* sp. Ar 6	-	11.0	Thermotolerant
46	*Penicillium* sp. Ar 12	0.58	18.0	Mesophile
47	*Penicillium* sp. Sy 41	0.44	15.0	Mesophile
48	*Stachybotrys* sp. Hh 8	0.50	9.0	Alkalitolerant

*FP-filter paper

Basidial fungi collection strains were screened on their ability to synthetize laccase. 61 strains, representatives of *Eumycota* of different genera were applied in the study. These are: *Cerrena*, *Fomes*, *Ganoderma*, *Lentinus*, *Pleurotus*, *Panus*, *Piptoporus*, *Trametes*and *Pseudotrametes*.

As a result of screening 21 strains of the basidial fungi were revealed as laccase producers (Table 2).

For the optimization of biosynthesis of cellulose degrading enzymes - cellulases and xylanases submerge cultivation of microscopic fungi was conducted in nutrient media containing various carbon sources: hay, malt sprouts, wheat bran, tea and wine production wastes, magazine paper, filter paper, microcrystalline cellulose and carboxymethyl cellulose at concentration 1%. Disaccharides and monosaccharides, in particular lactose, arabinose, mannitol, sucrose, rhamnose, sorbitol, xylose, galoctose, glycerol and glucose at 0.8% concentration according to carbon, were also applied as carbon sources.

The highest cellulase activity was observed while growing of studied fungi strains in microcrystalline cellulose containing medium. High xylanase activity was observed when xylan and soybean flour (SF) were included in the cultivation medium (Table 3).

Table 2. Laccase activities of basidial fungi strains

# No	Strains	Laccase, U/l	
		6 days	12 days
1	*Ganodermalucidum*GM -04	960	1686
2	*Ganodermalucidum*IG-74	-	1120
3	*Lentinus edodes* GK-97	406	156
4	*Panus tigrinus* GK-39	182	3584
5	*Pleurotusostreotus* GV-12	290	2436
6	*Pleurotusostreotus* GK-10	89	300
7	*PleurotusOstreotus* GK-52	130	246
8	*Fomes fomentarius* GK-33	0	4 256
9	*Piptoporusbetulinus*IK-25	562	55
10	*Trametesmaxima* GK-15	b	4 200
11	*Pseudotrametesgibbosa*IK-76	651	12 600
12	*Ganodermaapplanatum*IN-59	3 605	11 984
13	*Pleurotusostreotus* GD-41	123	424
14	*Pleurotusostreotus* IN-22	20	127
15	*Pleurotusdrynus*IN- 11	20 160	39 760
16	*Trameteshirsutus*GN 08	950	9 548
17	*Ganodermaapplanatum* IN-18	8 984	13 984
18	*Ganodermaadspersum* IN-58	5 135	9 296
19	*GanodermaSp* GV -01	57 200	98 880
20	*Piptoporusbetulinus*IK-26	180	652
21	*Trametesmaxima* GK-02	32 400	24 00

In another experiments nutrient media with different nitrogen and phosporus sources: NaH_2PO_4; $(NH_4)_2HPO_4$; KH_2PO_4; K_2HPO_4 were used for fungi cultivation.

As a result, optimum liquid nutrient media of fungi cultivation for the best enzyme production have been established. Below are the composition of these media, which were applied in further studies.

Medium for the production of cellulases, in %: microcrystalline cellulose – 0.1; $NaNO_3$ – 0,.; KH_2PO_4 - 0.2; $MgSO_4$ x$7H_2O$ - 0.05; maize extract – 1.5.

Medium for the production of xylanase, in %: soybean flour 3.0; $NaNO_3$ – 0.24; $(NH_4)_2SO_4$ – 0.2; KCl - 0.05; $MgSO_4$ – 0.015.

Results show that optimum temperature of growth for mesophilic strains varies between 27-32^{0}C, for thermotolerants - between 35-40^{0}C, and for thermophiles between 40-45^{0}C.

As for pH, optimum value for fungi submerged cultivation for mesophilic (by pH) strains varies between 5.0-6.5 pH, for alkalitolerant strains between pH 6.5-8.5 and for acidotolerants, between pH 2.5-5.5.

Table 3. Cellulase and xylanase activitiesof microscopic fungi strains grown on optimum carbon sources substrates

Strains	Cellulase FPA, U /ml		Xylanase, U/ ml	
	Substrates			
	MCC (1 %)	MCC(2 %)	Xylan	SF
Aspergillus niger A 7-5	-	-	20.0	18.5

*Aspergillus niger*Aj 38	-	-	30.0	25.0
Penicillium canescence D85	1.05	0.88	24.0	32.0
Sporotrichum pulverulentum T 5-0	0.84	0.75	9.0	7.5
Trichoderma viride X33	0.94	1.0	-	-
Aspergillus sp. Av 10	0.80	0.96	7.5	8.0

FPA - filter paper activity; MCC – micro crystalline cellulose, SF - soybean flour

Table 4. Strains of microscopic fungi, active producers of Cellulase and xylanase

#	Culture	Cellulase activity, (*FP) U/ml	Xylanase activity, U/ml	Characterization
1	*Aspergillus niger* A 7-5	-	20.0	Thermotolerant
2	*Aspergillus niger* Aj 38	trace	25.0	Thermotolerant
3	*Aspergillus*sp. Av 10	0.80	8.0	Alkalitolerant Thermotolerant Moderate halophile
4	*Penicillium canescence* D85	1.05	32.0	Acidotolerant
5	*Sporotrichum pulverulentum* T5-0	0.84	12.0	Thermophile
6	*Trichoderma viride* X33	0.94	trace	Alkalitolerant
7	*Aspergillusniger* H 6-1	-	25.0	Mesophile
8	*Aspergillus terreus* V2-8	0.70	3.2	ThermotolerantAlkalitolerant
9	*Chaetomium*sp.Y 3-1	0.84	9.0	Thermotolerant
10	*Penicillium* sp. Y 1-2	0.80	7.5	Mesophile
11	*Fusarium*sp. Z 7-1	0.58	-	Psychrotolerant

For the optimization of nutrient medium for laccase producer basidial fungi strains, various carbon sources at 1.5% concentration have been applied in initial synthetic medium during the submerged fermentation. The highest laccase activity among the enzyme producer GK 52,were obtained in medium with glycerol ; glycerol was shown as the best carbon source also for *Trametes maxima* GK-15, *Pseudotrametes gibbosa* IK-76, *Ganoderma applanatum* IN-59 and *Pleurotus drynus* IN- 11. Comparatively high laccase activity was detected in glucose containing medium. Some carbon containing substrates appeared ineffective for laccase biosynthesis; For strains: *Ganoderma lucidum* GM 04, *Pleurotus ostreatus* GK 52 - it was avicel; for strains *Trametes maxima* GK-15, *Pseudotrametes gibbosa*IK-76 - xylan; as for strains –

Ganoderma applanatum IN-59 and *Pleurotus drynus* IN-11-xylan and Na-gluconate.

Conclusions

Both microscopic and basidial fungi strains expressed different biosynthetic abilities of enzymes, depending on carbon, nitrogen and phosphorus sources, as well as on conditions of cultivation, such as aeration, temperature and pH. By optimization of nutrient media and cultivation conditions best cellulase and xylanase producer microscopic fungi strains, as well as best laccase producer basidial fungi strains have been selected.

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