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Higher Basidial Fungi Isolated from Different Zones of Georgia – Producers of Lignocellulosic Enzymes

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ABSTRACT. Great interest in basidiomycetes for targeted technological treatment of agro-industrial plant substrates is conditioned by their ability to produce lignocellulosic enzymes.

The samples of wood-degrading basidiomycetes were collected from different taxonomic niches of Georgia to study biochemistry and physiology of wood-degrading basidiomycetes. 36 strains were obtained as pure cultures and 29 ones were identified. Producers of lignocellulosic enzymes were revealed among the test fungi under solid-state and submerged cultivation conditions. *Pleurous ostreatus* GV12, *Pleurous ostreatus* GK10 and *Fomes.* sp. KA20 were found to be the best producers of cellulosic enzymes using orange peel, wheat straw and wheat bran as substrates, and *Ganoderma* sp. GM 04 – the best producer of laccase during cultivation on orange peel waste. The influence of lignocellulose on accumulation of the enzymes laccase, xylanase and filter paper assay was studied. *© 2014 Bull. Georg. Natl. Acad. Sci.*

Key words: *lignocellulosic materials, basidiomycetes, laccase, cellulase, xylanase.*

Introduction

Large amount of plant wastes of agricultural and food-canning industries that are formed in the world make great ecological threats to population. In the last decade the interest in application of fungi potential for biotechnological processes is significantly increased [1, 2]. Filamentous fungi is a special group of organisms capable of degrading lignocellose and consequently forming many biologically active compounds [3, 4]. In the mentioned processes, the role of white rot basidial fungi capable to synthesize hydrolyzing and oxidizing enzymes, which play an important role in degradation of polysaccharides and lignin of plant biomass, should be especially noted. Delignified natural wastes are one of the most considerable materials for production of bioethanol. Hence, lignocellulose-degrading enzymes are one of the most significant compounds for bioconversion of plant wastes [5, 4]. For efficient development of microbial technologies, selection of proper plant raw materials,

on which synthesis of targeted enzymes occur more effectively, is of main importance for cultivation of fungi [6, 7]. Thus, the conducted work was principally directed at enzymatic hydrolysis of lignocellulose, taking into consideration that treatment of lignocellulose by white rot fungi decreases resistance of substrate with enzymatic hydrolysis and improves technological processes of energy production.

MATERIALS AND METHODS

Lignocellulosic substrates. Wheat bran, orange peelings and wheat straw are plant wastes available at the local market and bagasse existent at the international market were applied in the studies. All residues were dried at 50°C and milled to dust extent (<1mm).

Microorganisms identification. Isolation of basidial fungi was performed in two ways: isolation of cultures from basidiospores and carposomes. Then the sample was placed on a sterile glass slide with hymeneal layer down. As a result, spore reflection was obtained. Spore dust was put into water and diluted. The obtained inoculant was reinoculated on solid medium of the following composition: malt extract – 5%; agar – 2%; pH – 5.8–6.5. Basidial cultures were grown in thermostat at 28 °C. Morphological description, physiological, biochemical and electromicroscopic methods were applied for identification of basidiomycetes. For identification of basidial fungi the manuals by Nobles and Bukhalo [8, 9] were used.

Fungal inocula were prepared by growing the fungi on a rotary shaker at 180 rpm, 27°C in 500 ml flasks containing 100 ml of synthetic medium with the following composition (g/l): glucose – 15.0; ammonium nitrate – 3.0; yeast extract – 3.0; NaH_2PO_4 – 0.9; K_2HPO_4 – 0.3; $MgSO_4$ – 0.5; initial pH was adjusted to 5.7 prior to sterilization. The nutrient medium was sterilized at 121°C for 20 min. After 7-10 days of fungi cultivation, mycelium was inoculated to conduct the SSF and SF of selected lignocellulosic materials.

Cultivation conditions. Solid-state fermentation (SSF) of the selected plant residues was car-ried out at 27 °C in 100-ml flasks containing 5 g of lignocellulosic substrates moistened with 18 ml of the nutrient medium (g/l): $NaNO_3$ – 2.0; yeast extract – 3.0; NaH_2PO_4 – 0.9; K_2HPO_4 – 0.3; $MgSO_4 \times 7H_2O$ – 0.5; 0.2 mM $CuSO_4 \times 5H_2O$; pH 5.8. The flasks were inoculated with 5 ml of mycelial homogenate. After 7 and 15 days of cultivation the extracellular enzymes were extracted from the whole biomass twice with 25 ml of distilled water (total volume 50 ml). The extract was centrifuged at 10000 g for 15 min at 4 °C. Filtrate was S used for determination of enzyme activities.

Submerged fermentation (SF) of fungi was performed at 27°C on a rotary shaker at 180 rpm in 500-ml flasks containing 100 ml of the above-mentioned medium, (g/l): Lignocellulosic substrate – 5%; $NaNO_3$ –3.0; yeast extract – 2.0; NaH_2PO_4 – 0.9; K_2HPO_4 – 0.3; $MgSO_4 \times 7H_2O$ – 0.5; 0.2 mM $CuSO_4 \times 5H_2O$; Initial pH was adjusted to 5.8. The flask was sterilized at 121°C for 35 min. The flasks were inoculated with 10 ml of mycelial ho-mogenate. On the 6th and 10th days of cultivation, the culture liquid was centrifuged at 10000 g for 15 min at 4 °C and filtrate used for determination of enzyme activities.

Enzyme Assays. Carboxymethyl cellulase (CMCase) activity was assayed according to IUPAC recommendations by mixing 100 µl appropriately diluted samples with 100 µl of low-viscosity carboxymethyl cellulose (1% w/v) in 50 mM citrate buffer (pH 5.0) at 50°C for 10 min [10].

Xylanase activity was determined by mixing 70 µl of appropriately diluted samples with 630 µl of birch wood xylan (Roth 7500) (1% w/v) in 50 mM citrate buffer (pH 5.0) at 50°C for 10 min [11]. Glucose and xylose standard curves were used to calculate cellulase and xylanase activities. In all the assays the release of reducing sugars was measured using the dinitrosalicylic acid reagent method [12].

Filter paper assay for saccharifying cellulase (FPA) activity was assayed using Whatman filter paper №1 according to IUPAC recommendations [10]. One unit of enzymes activity was defined as the amount of enzymes releasing 1 µmol of reducing sugars per minute.

Laccase activity was determined by monitoring spectrophotometrically the change in absorbance at 420 nm (A420) related to the rate of oxidation of 1 mM 2,20-azino-bis-[3-ethylbenz-thiazoline-6-sulfonate] (ABTS) in 50 mM Na-acetate buffer (pH 3.8) [13]. Assays were performed in 1-ml cuvettes at room temperature with 50 μl of adequately diluted culture liquid.

Results and Discussion

Basidiomycetes enzyme activity in solid-state fermentation (Screening).

To estimate the enzyme production, basidial fungi were screened during their cultivation on wheat straw. The mentioned substrate is a complex plant waste containing all those polymers (cellulose, hemicellulose, lignin), degradation ability of which possess higher filamentous fungi. 21 strains of different genera and species were chosen for testing, At SSF testing of basiodiomycetes on wheat straw substrate, their various potentials were revealed in terms of accumulation of both hydrolyzing (CMCase, xylanase, FPA) and oxidizing (laccase) enzymes (Table 1).

It is known that enzyme synthesis by microorganisms significantly depends on the time period and conditions of cultivation [14,15]. Thus, activities of synthesized enzymes were tested on the 6th, 15th and 20th days of cultivation. The indices varied in rather wide range: Laccase – 53-2096 U/l, CMCase – 1.3-16.4 U/ml, xylanase – 1.5-25.3 U/ml, FPA – 0.05-0.52 U/ml. *Pleurotus ostreatus* GK10 was found to be the best producer of hydrolyzing enzymes (CMCase, xylanase, FPA). Enzyme activities were 16.4 U/ml, 25.3 U/ml and 0.52 U/ml, respectively. However, the same strain displayed rather high laccase activity – 1135 U/l. Fungi *Fomes*. sp. KA20 and *Pleurotus* sp. GD41 also revealed high ability to synthesize all four test enzymes. The strains *Pleurotus ostreatus* GV12 and *Fomitopsis pinicola* IK45 showed high ability to accumulate hydrolases only. It should be mentioned that revelation of producers of certain enzymes is of importance for technological processes [16].

As is seen in Table 1, all fungi displayed the ability to synthesize laccase on wheat straw substrate, though, the fungal strain *Ganoderma* sp. GM 04 revealed the highest activity. High ability to synthesize laccase were also displayed by the strains

Table 1. Basidiomycetes enzyme activity in solid-state fermentation of wheat straw

Fungus	Final pH	Laccase, U/ l	CMCase, U /ml	Xylanase, U /ml	FPA, U/ ml
Fomes fomentarius GK93	4.8	360±56	3.9±0.3	5.3±0.4	0.14±0.02
Fomes fomentarius GK33	5.3	920±122	3.0±0.2	6.9±1.1	0.22±0.05
Fomes sp. KA 35	4.9	298±39	6.0±0.5	7.9±0.7	0.23±0.07
Fomes.sp. KA20	4.9	1110±201	11.8±0.5	19.2±1.2	0.40±0.03
Fomitopsis pinicola IK45	5.9	53±11	13.8±0.2	16.8±0.7	0.36±0.11
Fomitopsis sp. IK46	4.8	202±48	3.9±0.3	4.3±0.2	0.19±0.05
Ganoderma applanatum IN18	6.3	100±22	9.2±1.9	8.4±1.2	0.25±0.10
Ganoderma sp. GM 04	5.5	2096±218	4.0±0.6	6.1±1.4	0.17±0.05
Ganoderma lucidum IG74	5.1	1068±230	3.5±0.1	5.3±0.4	0.09±0.02
Ganoderma sp. IN59	4.5	910±145	2.2±0.3	1.5±0.1	0.09±0.02
Lentinus edodes GK97	6.0	61±15	1.3±0.2	0.44±0.1	0.05±0.01
Pleurotus ostreatus IN22	5.2	353±77	5.2±0.5	2.5.±0.6	0.11±0.03
Pleurotus ostreatus GV12	5.4	271±64	14.2±1.6	23.1±1.5	0.45±0.05
Pleurotus ostreatus GK10	5.9	1135±185	16.4±2.2	25.3±1.3	0.52±0.08
Pleurotus ostreatus GK52	5.5	1055±65	4.6±0.4	6.8±0.8	0.11±0.05
Pleurotus sp. GD41	5.5	966±33	12.4±0.4	19.6±1.3	0.34±0.03
*Trametes sp.*GK 68	4.2	560±165	1.6±0.4	4.0±0.1	0.05±0.01
Sabaduri 1	5.4	1019±222	5.1±0.5	3.8±0.1	0.20±0.06
Sabaduri 4	5.2	829±235	4.7±0.4	6.2±0.3	0.28±0.05
Shovi 9	5.6	530±143	1.4±0.3	4.8±0.4	0.13±0.04
Kutaisi 7	4.4	165±37	8.1±0.7	11.8±0.6	0.41±0.02

Table 2. Effect of lignocellulosic substrates on the basidiomycetes' hydrolytic enzymes activity (SSF)

Fungi	Substrates	Final pH	Xylanase U/ml	FPA U/ml
Pleurous ostreatus GK10				
	Orange peel	4.9	22.1	0.48
	Wheat bran	5.6	1 9.1	0.41
	Wheat straw	5.9	24.9	0.53
	Bagassa	5.0	2.1	0.04
	Sawdust	4.8	7.2	0.21
Pleurous ostreatus GV12				
	Orange peel	4.8	12.8	0.35
	Wheat bran	5.3	17.9	0.47
	Wheat straw	5.4	22.8	0.43
	Bagassa	4.8	1.3	0.04
	Sawdust	4.9	8.0	0.23
Fomes.sp. KA20				
	Orange peel	5.0	17.3	0.60
	Wheat bran	6.1	21.7	0.52
	Wheat straw	4.8	18.7	0.45
	Bagassa	4.7	2.2	0.05
	Sawdust	5.0	19.9	0.44
Ganoderma sp. GM 04				
	Orange peel	3.9	5.9	0.20
	Wheat bran	5.4	4.2	0.15
	Wheat straw	5.2	7.8	0.18
	Bagassa	5.3	0.9	0.14
	Sawdust	5.2	8.1	0.21

Pleurotus ostreatus GK10, *Fomes* sp. KA20 and *Ganoderma lucidum* IG 74. It is noteworthy that fungi in the range of the same genus revealed different activities of enzymes: *e.g. Pleurotus ostreatus* GK10 displayed high ability to accumulate all four test enzymes; *Pleurotus ostreatus* GV12, representative of the same genus was distinguished by synthesis of hydrolases only and *Pleurotus ostreatus* GK52 showed high laccase activity.

Thus, according to the results of screening and literary data [17, 4], the unique property of basidial fungi is to synthesize simultaneously both hydrolyzing and oxidizing enzymes on lignocellulosic material. New isolates of fungi: *Pleurotus ostreatus* GK10, *Pleurotus ostreatus* GV12, *Fomes.*sp. KA20 and *Ganoderma* sp. GM 04 are rather prospective strains in terms of both obtaining lignocellulosic enzymatic preparations and delignification of waste plant material.

The effect of lignocellulosic substrates on the basidiomycetes hydrolytic enzymes activity

Hydrolyzing enzymes play an important role in degradation of polysaccharides of plant wastes to low-molecular compounds [18, 19]. In this point of view, white rot higher filamentous fungi are significant organisms, since they are able to synthesize all those hydrolyzing enzymes (endo-1,4-ß-D-glucanase (EC 3.2.1.4), exo-1,4-ß-D-glucanase (EC 3.2.1.91) and xylanase (EC 3.2.1.8), which may be applied in different biotechnological processes based on conversion of plant biomass. Synthesis of the mentioned enzymes significantly depend on mode and time of cultivation. The influence of lignocelluloses on the selected strains under SSF and SF cultivation conditions was studied (Table 2, 3). In case of all four test strains SF appeared to be the best condition for accumulation of hydrolases, in which production of test enzymes was increased by 2-3 folds. In case of *Fomes* sp. KA20, if under SSF cultivation conditions, activity of xylanase and FPA activity on orange peel were 17.3-0.60 U/ml, and under SF conditions, these indices were increased up to 36.6-1.15 U/ml, respectively. Similar action was observed in case of the other strains and substrates. However, for sadwust, solid-phase fermentation was found to be prospective. It should be mentioned that for accumulation of en-

Table 3. Effect of lignocellulosic substrates on the basidiomycetes' hydrolytic enzymes activity (SF)

Fungi	*Substrates*	*Final pH*	*Xylanase U/ml*	*FPA U/ml*
Pleurous ostreatus GK10				
	Orange peel	5.0	26.7	0.71
	Wheat bran	5.2	24.5	0.69
	Wheat straw	5.3	28.5	0.71
	Bagassa	5.6	3.2	0.10
	Sawdust	4.9	5.2	0.13
Pleurous ostreatus GV12				
	Orange peel	5.1	31.1	0.90
	Wheat bran	5.8	29.0	0.82
	Wheat straw	5.9	27.3	0.73
	Bagassa	6.0	6.2	0.19
	Sawdust	5.0	4.0	0.15
Fomes.sp. KA20				
	Orange peel	5.0	36.6	1.15
	Wheat bran	5.7	31.8	1.00
	Wheat straw	5.3	28.1	0.62
	Bagassa	5.5	9.1	0.28
	Sawdust	5.0	6.9	0.18
Ganoderma sp. GM 04				
	Orange peel	4.8	16.3	0.44
	Wheat bran	5.3	15.5	0.35
	Wheat straw	5.4	11.6	0.27
	Bagassa	5.8	5.0	0.18
	Sawdust	4.9	6.3	0.17

zymes, in both types of cultivation, orange peel was the best and then wheat straw and wheat bran. Hence, the process of delignification in the mentioned plant substrates is the issue of further investigations.

The effect of lignocellulosic substrates on the basidiomycetes' laccase activity

Existence of lignin barrier is the main obstacle for obtaining the product with additional value during lignocellulase conversion [21, 22]. Besides, it is known that some plant waste significantly stimulates secretion of ligninase enzymes without adding specific inductors [23-25].

White rot basidiomycetes possess necessary extracellular lignocellulosic enzymatic system, including that of laccase (EC 1.10.3.2) participating in degradation of lignin during cultivation under SSF and

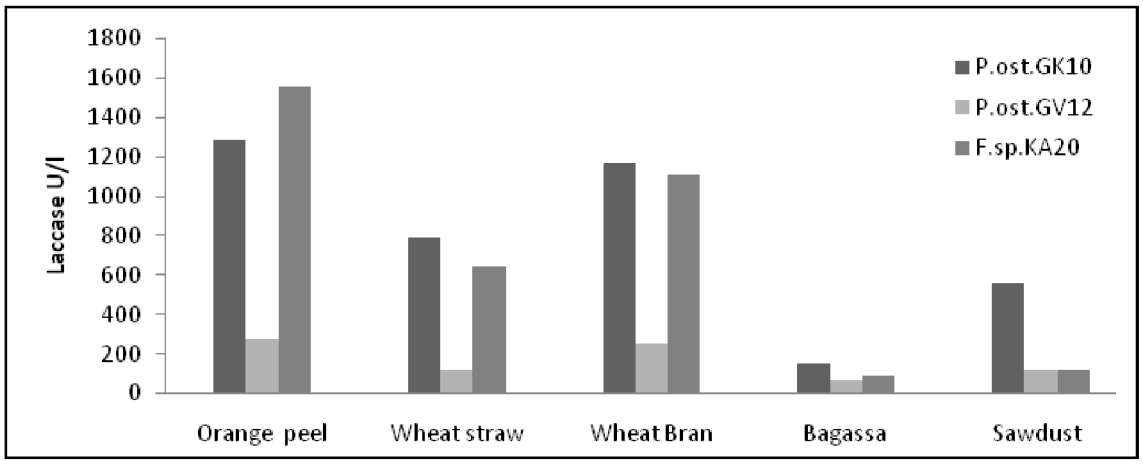

Fig. 1. Effect of lignocellulosic substrates on the basidiomycetes' laccase activity SSF

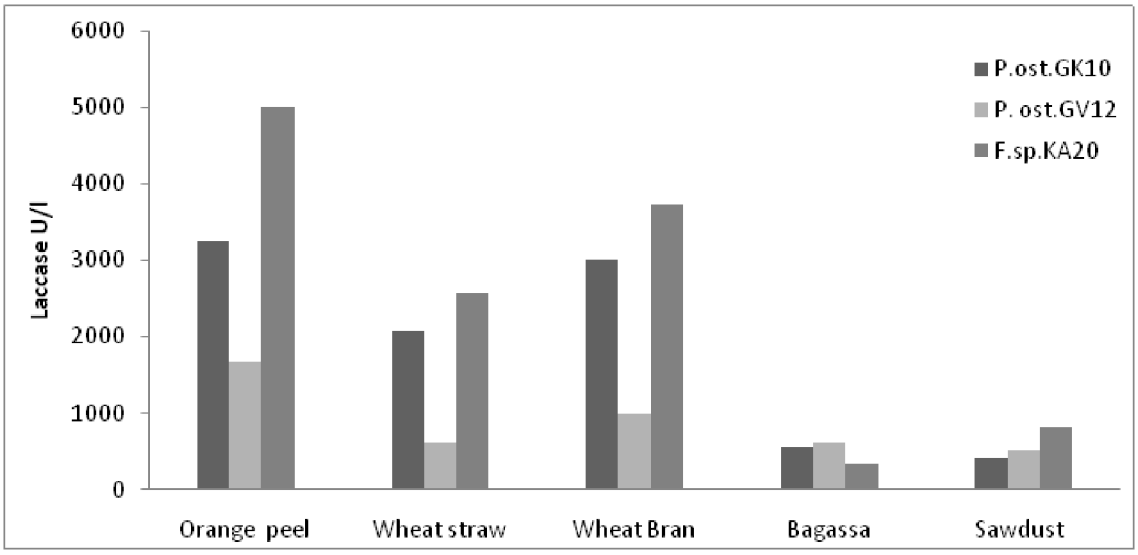

Fig. 2. Effect of lignocellulosic substrates on the basidiomycetes' laccase activity SF

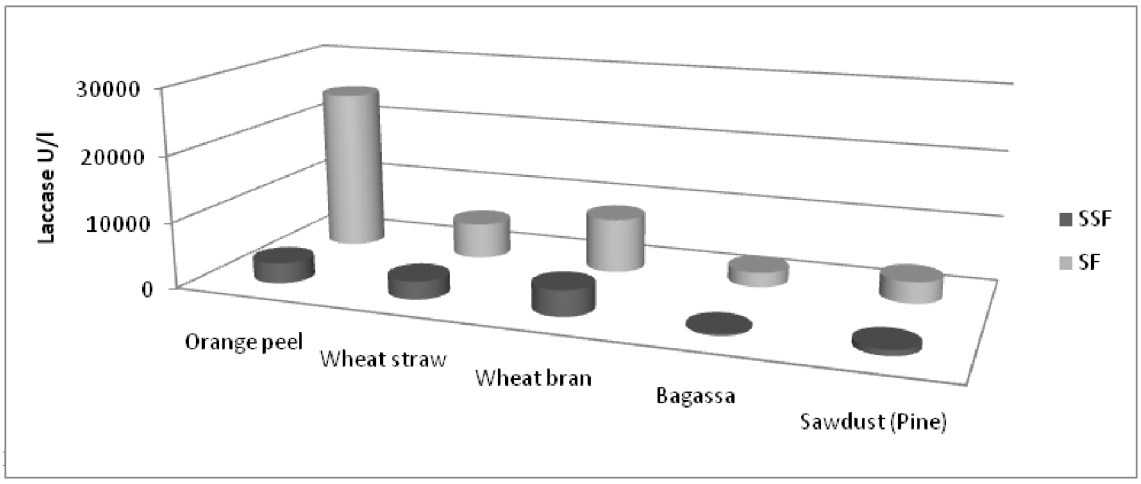

Fig. 3. Effect of ligrocellulosic substrates on the *Ganoderma* sp. GM04 Laccase activity SSF and SF

SF conditions. The influence of lignocellulose on secretion of the mentioned enzyme was studied in selected strains in the series of experiments (Figs.1,2). The best condition for accumulation of laccase for all the test strains was SF, when the index of laccase activity was significantly higher in comparison with that of SSF. Similar to hydrolyzing enzymes, production of laccase occurred more efficiently in orange peel, wheat bran, and wheat straw substrates. The activity index varied in rather wide range from 67.8 to 24 640.0 U/l, depending on cultivation conditions and on species of fungi. *Ganoderma* sp. GM 04 (Fig.3) appeared to be rather prospective strain for production of laccase. It displayed more or less laccase-synthesizing ability on all test lignocelluloses. The best activity was shown on orange peel.

Conducted studies show that selection of plant biomass for production of target enzyme is of special importance playing decisive role in effective development of technologies.

ბიოტექნოლოგია

საქართველოს სხვადასხვა რეგიონებიდან გამოყოფილი ლიგნოცელულაზური ფერმენტების პროდუცენტი - უმაღლესი ბაზიდიალური სოკოები

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აგროინდუსტრიული მცენარეული სუბსტრატების მიზნობრივ ტექნოლოგიურ დამუშავებაში ბაზიდიომიცეტებისადმი დიდ ინტერესს განაპირობებს მათი ლიგნოცელულაზური ფერმენტების პროდუცირების უნარი. ხის დამშლელი ბაზიდიომიცეტების ბიოქიმიისა და ფიზიოლოგიის შესწავლის მიზნით საქართველოს სხვადასხვა ეკოლოგიური რეგიონიდან გამოყოფილია ხის დამშლელი ბაზიდიომიცეტების კულტურები. სუფთა კულტურის სახით მიღებულია 36, ხოლო იდენტიფიცირებულია 29 შტამი. ტესტირებულ სოკოებში გამოვლენილ იქნა ლიგნოცელულაზური ფერმენტების პროდუცენტები მყარფაზოვანი და სიღრმული კულტივირების პირობებში. ფორთოხლის, ნამჯის, ქატოს სუბსტრატებზე ცელულაზური ფერმენტების საუკეთესო პროდუცენტებია *Pleurous ostreatus GV12, Pleurous ostreatus GK10* და *Fomes.sp. KA20*, კულტურა – *Ganoderma sp. GM 04* მაღალ ლაკაზურ აქტივობას იძლევა ფორთოხლის ნარჩენზე კულტივირებისას. შესწავლილია სხვადასხვა ლიგნოცელულოზური სუბსტრატის გავლენა ფერმენტების ლაკაზას, ქსილანაზასა და ცელულაზას დაგროვებაზე.

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