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Collection of Microorganisms to Create a Consortium for the Biodegradation of Keratin-Containing Raw Materials from Poultry Industry Waste

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Abstract

Biodegradable ability of microorganisms, biomass accumulation rates, mass fraction of protein, degree of hydrolysis of keratin, keratin hydrolyzate digestibility *in vitro*, keratinase optimal activity at each of these conditions and duration of culture growth, and biochemical and phenotypic properties of the microorganisms are studied. The selection of microorganisms to create a consortium for processing of keratin materials is explained. The conditions for the biodestruction of poultry industry waste by the consortium are determined.

Keywords

Microorganisms; Keratin-containing raw material; Consortium

Introduction

The most promising sector of global and Russian agriculture is poultry. Nowadays, poultry meat is second only to pork in the world meat market [1].

The share of the poultry industry in meat production in Russia in 2012 was 3,587 thousand tons. The share of poultry meat in the total meat production reached 42% versus 18%—in 1990, which corresponds to the global trends. The growing production of poultry meat significantly increases the volume of waste. For example, in 2012 the volume of waste of poultry processing amounted 1.2 million tons [2].

At the same time, the most interesting of all waste of poultry evisceration is feather and downy raw materials because it makes 30% of the total waste. The basic protein that is part of these raw materials is keratin [3-6].

Currently, special attention is paid to the choice of the direction of utilization, where the priority is mainly given to the process aimed at extracting useful components from waste [7-9].

Also, known processing technologies of feather and downy raw materials by bioconversion are characterized by a number of disadvantages: low degree of bioconversion, low yield of protein, multistage and time-consuming process, etc. [10-12]. In this connection, there is a need to improve bioconversion technology, for a better understanding of the fundamental aspects of the processes underlying the biocatalytic conversion of components of keratin-containing waste, and to use new knowledge for the development of new technological solutions [13, 14].

The objective of this work is the screening of a collection of microorganisms-destructors in order to create a consortium for the processing of keratin materials.

Materials and Methods

Materials

Theoretical and experimental studies were carried out in accordance with the tasks performed at the Scientific and Educational Center and the Department of Bionanotechnology of the Kemerovo Institute of Food Science and Technology.

As producers of enzymes with keratinase action, 15 strains (reflected in Table 1) provided by GosNIIgenetika were used (http://www.genetika.ru/vkpm).

Determination of the mass fraction of total protein

The mass fraction of total protein was determined by Dumas method, which is based on measuring the thermal conductivity of the molecular nitrogen formed after burning the test sample at a temperature of about 1000°C in an atmosphere of oxygen and subsequent reduction of the nitrogen oxides using a reducing agent (copper) with a nitrogen analyzer protein, RAPID N Cube. Before combustion, the test solution was placed in a special tin foil capsule that is nitrogen-free, and then in the cell analyzer autosampler of protein nitrogen RAPID N Cube.

Determination of keratinase activity

Keratinase activity was determined by a modified method. To determine the keratinase activity (KA), we took 200 mg of crushed feathers (previously washed with chloroform and water, and air-dried) and 10 ml of 0.05 M borate buffer (pH 9.0) containing an amount of enzyme equivalent to 0.02 units of proteolytic activity (PA). Then it was energetically shaken and left for 3 h at 37°C for the hydrolysis of keratin. Simultaneously, two controls were set: dissolution buffer and feathers in soluble protein in the culture medium.

Upon completion of hydrolysis, the remaining undigested protein was precipitated by a solution with trichloroacetic acid and filtered. The absorbance in the filtrate was measured at a wavelength of 340 nm.

A calibration curve constructed based on the serum albumin solution, determined the amount of digested protein (g/cm^3) in the culture medium for 1 h hydrolysis.

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No.	Genus	Species	Number of strain in the "GosNIIgenetika" collection
1	*Bacillus*	*Bacillus licheniformis*	B-10469
2	*Bacillus*	*Bacillus licheniformis*	B-2986
3	*Bacillus*	*Bacillus licheniformis*	B-2708
4	*Bacillus*	*Bacillus coagulans*	B-9868
5	*Streptomyces*	*Streptomyces fradiae*	AC-1330
6	*Streptomyces*	*Streptomyces ornatus*	S-1220
7	*Streptomyces*	*Streptomyces rimosus*	AC-324
8	*Streptomyces*	*Streptomyces parvus*	AC-1114
9	*Streptomyces*	*Streptomyces parvus*	AC-1116
10	*Streptomyces*	*Streptomyces griseus*	AC-293
11	*Penicillium*	*Penicillium rubrum*	F-601
12	*Verticillium*	*Verticillium lateritum*	F-626
13	*Verticillium*	*Verticillium lecanii*	F-239
14	*Aspergillus*	*Aspergillus foetidus*	F-406
15	*Aspergillus*	*Aspergillus oryzae*	F-1008

Table 1: Microorganisms-destructors of keratin-containing wastes

Specific activity of the enzyme is a number of activity units referred to 1 mg of protein in enzyme preparation.

Determination of the degree of hydrolysis

The degree of hydrolysis was determined as the ratio of amino nitrogen to total nitrogen.

Determination of the antibiotic properties of microorganisms

Determination of antibiotic properties of microorganisms on solid medium was performed by histogram. The culture of the test strain was inoculated by a stroke into Petri dishes on a nutrient medium and incubated at optimal temperature for 2 h to form a diffusion in agar and inhibitory compounds. Then exponential culture of the test strain was co-inoculated (*Escherichia coli* or *Staphylococcus aureus*). The dish was again incubated at 37°C for 2 h. When determining the antimicrobial activity of the microorganisms being tested by blocks, a deep culture was grown in a nutrient-agar method in a Petri dish and incubated at optimal temperature for 24 h. Then the agar disk (block) with the grown culture was cut using a sterile mirror drill and installed in another Petri dish on the surface of the agar medium, just inoculated by the test strain (*Escherichia coli* or *Staphylococcus aureus*). The dish was incubated at 37°C for 36 h.

The conclusion on the antibiotic activity of microorganisms was based on the width of the zone of inhibition.

Determination of phenotypic properties of microorganisms

Phenotypic properties of microorganisms were evaluated by microscopy.

Determination of the biochemical properties of microorganisms

The biochemical properties of these strains were analyzed by a standardized test system, API 50 CHB, with the identification software Apiweb produced by VioMerieux (France). API 50 CHB is a research strip used to determine the metabolism of sugars by various microorganisms using three different media and that allows defining up to 170 taxa. The working principle of API 50 CHB is that during incubation, the implementation of metabolism of studied objects pH shift occurs, which is accompanied by a change in color of the substrate in the wells of the test strips. To change the reaction color is evaluated as positive or negative.

No.	Species	Number of strain in the "GosNIIgenetika" collection	Optimal temperature, °C	Optimal pH
1	*Bacillus licheniformis*	B-10469	37	9.0-10.0
2	*Bacillus licheniformis*	B-2986	37	6.8-7.0
3	*Bacillus licheniformis*	B-2708	37	6.8-7.0
4	*Bacillus coagulans*	B-9868	40	6.8-7.0
5	*Streptomyces fradiae*	AC-1330	28	7.0-7.5
6	*Streptomyces ornatus*	S-1220	30	7.0-7.2
7	*Streptomyces rimosus*	AC-324	28	7.0-7.2
8	*Streptomyces parvus*	AC-1114	28	7.0-.2
9	*Streptomyces parvus*	AC-1116	28	7.0-7.2
10	*Streptomyces griseus*	AC-293	28	9.0-10.0
11	*Penicillium rubrum*	F-601	24	6.0-6.5
12	*Verticillium lateritum*	F-626	25	7.0-7.5
13	*Verticillium lecanii*	F-239	24	7.0-7.2
14	*Aspergillus foetidus*	F-406	32	5.0-5.5
15	*Aspergillus oryzae*	F-1008	25	7.0-7.2

Table 2: Strains of microorganisms for the study of keratinase activity

Statistical analysis

All repeated experiments were performed three times. Data processing was carried out by standard methods of mathematical statistics.

Results

Fifteen strains with KA were selected to study the biodegradation ability (Table 2).

The following indicators of the examined strains were studied at optimal growth conditions for each during the 12.0 h of cultivation: biomass accumulation, mass fraction of protein, degree of hydrolysis of keratin, keratin hydrolyzate digestibility *in vitro*, KA (Table 3).

Phenotypic characteristics and biochemical properties of the selected microbial strain with KA were also studied.

The results show that inoculation of strain *Bacillus licheniformis* B-2986 on a solid growth medium (g/l distilled water: meat water—1.0l; sodium chloride—5.0; peptone—10.0; agar—20.0) with an optimum cultivation temperature of 37°C and pH 6.8-7.0 results in the formation of bacteria that are nontransparent and rough and that grow into the agar colony. Bacteria are of $0.5 \times 2.0 \times 3.0\ \mu$m size and have the form of single rods, rare of short chains; cells are motile and Gram positive. The culture produces spores that have a central position and an oval shape.

Strain	Indicator value				
	Accumulation of biomass, CFU/g ·10^{-3}	Mass fraction protein in biomass, %	Degree of hydrolysis, %	Keratinase activity, U/mg of protein	Keratin hydrolyzate digestibility *in vitro*, %
Bacillus licheniformis B-10469	204.0 ± 12.2	44.09 ± 3.09	49.97 ± 2.50	12.0 ± 0.6	55.2 ± 2.8
***Bacillus licheniformis* B-2986**	**280.0 ± 16.8**	**64.02 ± 4.48**	**56.78 ± 2.84**	**14.6 ± 0.7**	**67.4 ± 3.4**
Bacillus licheniformis B-2708	98.0 ± 5.9	24.54 ± 1.72	21.12 ± 1.06	7.5 ± 0.4	39.3 ± 1.9
Bacillus coagulans B-9868	108.0 ± 6.5	26.55 ± 1.86	24.87 ± 1.24	8.9 ± 0.4	42.0 ± 2.1
Streptomyces fradiae AC-1330	120.0 ± 7.2	29.25 ± 2.05	36.78 ± 1.84	11.4 ± 0.6	45.6 ± 2.3
***Streptomyces ornatus* S-1220**	**430.0 ± 25.8**	**85.44 ± 5.98**	**68.96 ± 3.45**	**32.4 ± 1.6**	**78.2 ± 3.9**
Streptomyces rimosus AC-324	145.0 ± 8.7	32.09 ± 2.25	41.23 ± 2.06	12.2 ± 0.6	47.7 ± 2.4
Streptomyces parvus AC-1114	114.0 ± 6.8	27.13 ± 1.90	33.08 ± 1.65	9.1 ± 0.5	44.1 ± 2.2
Streptomyces parvus AC-1116	175.0 ± 10.5	38.95 ± 2.73	52.34 ± 2.62	13.0 ± 0.7	50.2 ± 2.5
Streptomyces griseus AC-293	94.0 ± 5.6	22.09 ± 1.55	18.29 ± 0.91	7.2 ± 0.4	35.6 ± 1.8
***Penicillium rubrum* F-601**	**228.0 ± 13.7**	**63.12 ± 4.42**	**52.46 ± 2.62**	**21.6 ± 1.1**	**62.3 ± 3.1**
***Verticillium lateritum* F-626**	**342.0 ± 20.5**	**68.41 ± 4.79**	**62.34 ± 3.12**	**28.1 ± 1.4**	**75.3 ± 3.8**
Verticillium lecanii F-239	123.0 ± 7.4	31.04 ± 2.17	38.14 ± 1.91	9.5 ± 0.5	46.3 ± 2.3
Aspergillus foetidus F-406	115.0 ± 6.9	26.32 ± 1.84	36.69 ± 1.85	9.0 ± 0.4	44.6 ± 2.2
Aspergillus oryzae F-1008	141.0 ± 8.5	30.80 ± 2.16	39.56 ± 1.98	11.9 ± 0.6	46.2 ± 2.3

Table 3: Properties of microorganisms exhibiting keratinase activity under optimum growth conditions during 12.0 h

When inoculating strain *Streptomyces ornatus* S-1220 on a solid growth medium (g/l distilled water: soluble starch—10.0; potassium hydrogen phosphate—1.0; magnesium sulfate—1.0; sodium chloride—1.0, ammonium sulfate—2.0; calcium carbonate—2.0; ferric sulfate—0.001; manganese chloride—0.001; zinc sulfate—0.001; agar—20.0) with an optimal cultivation temperature of 24°C and pH 6.8-7.2, the culture forms straight sporophores. According to electron microscopy analysis, the spores are rectangular, with a smooth surface. Aerial mycelium on synthetic media is from light gray, cream to pale yellow. Substrate mycelium is of yellowish-brown, golden-yellow to yellow.

When inoculating strain *Penicillium rubrum* F-601 on a dense nutrient medium (g/l of distilled water: potatoes—200.0; glucose—20.0; agar—20.0) with an optimal cultivation temperature of 24°C and pH 6.0-6.5, it forms colonies that rise in the central part, are furrowed and are gray-white, pale pink, grayish-green to green walnut. Conidiophores are short (50 × 1.5-2 μm), with branches of intertwined aerial hyphae; sometimes from hyphae embedded in agar they reach a length of up to 100 μm, are rarely branched and are smooth, with tassels on top, which consist of 8-20 sterigmata (6-7 × 1.5-2 μm). Conidia are spherical, slightly scabrous of 2-2.5 μm, and they quickly fall off from sterigmata. Reverzum is orange-red, hot pink, or brick-red, and agar is painted in the same colors, but less intensively.

When inoculating the strain *Verticillium lateritium* F-626 on a dense nutrient medium (g/l of distilled water: sodium nitrate—3.0; monopotassium phosphate—1.0; heptahydrate sulfate mineral epsomite—0.5; potassium chloride—0.5; iron (II) sulfate heptahydrate—0.01; sucrose—30.0; agar—20.0) with an optimal cultivation temperature of 25°C and pH 7.0-7.5, it forms a colony of diameter of 18-22 mm. Aerial mycelium is well developed, lush, fluffy, and white. Folding and radial zonation in colonies is almost not expressed. Fungal hyphae are thin, transparent, and septate, with conidiophores with phialides on which the conidia are formed. Conidia are numerous, oval, elongated, unicellular, and colorless at a length of 3-7 μm and width of 1.5-2 μm.

The biochemical properties of these strains were analyzed by a standardized test system, API 50 CHB, using the identification software Apiweb produced by VioMerieux (France). This test system comprises 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. The results obtained indicate that the strain *Bacillus licheniformis* B-2986 ferments glycerol, L-arabinose, D-xylose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, maltose, sucrose, trehalose, D-raffinose, starch, and glycogen.

Strain *Streptomyces ornatus* S-1220 grows well in the presence of L-arabinose, D-xylose, galactose, D-glucose, D-fructose, rhamnose, inositol, mannitol, maltose, lactose, sucrose, and starch.

Strain *Penicillium rubrum* F-601 ferments L-arabinose, L-xylose, D-glucose, inositol, mannitol, maltose, sucrose, starch, and xylitol.

Strain *Verticillium lateritium* F-626 exhibits substantial growth in the presence of L-arabinose, ribose, D-xylose, D-glucose, sorbitol, maltose, lactose, sucrose, and starch.

Then the resistance of these strains to antibiotics was studied. True natural resistance is characterized by the absence of target of the antibiotic or unavailability of the target due to primary low permeability or enzyme inactivation. Natural resistance is a constant feature of the species of microorganisms and easily predicted. In connection with this, the determination of the resistance of microorganisms is relevant as it will simplify the working with the strain in industrial scale.

To analyze the stability of the studied strains to antibiotics, the following antibiotics were used: chloramphenicol, streptomycin, tetracycline, kanamycin, penicillin. Obtained results are presented in Table 4.

Name of antibiotic	Concentration, %	Zone of inhibition of growth of bacteria, R, cm			
		Bacillus licheniformis B-2986	*Streptomyces ornatus* S-1220	*Penicillium rubrum* F-601	*Verticillium lateritum* F-626
Chloramphenicol	0.4	1.8	2.1	2.5	1.5
Streptomycin	0.4	2.0	1.8	1.4	2.3
Tetracycline	0.4	1.3	1.5	2.0	2.1
Kanamycin	0.4	1.5	2.4	1.9	1.9
Penicillin	0.4	2.3	1.6	1.6	2.0
Lysozyme	0.4	0.2	0.3	0.1	0.2
Control	0	–	–	–	–

Table 4: Indicators of antibiotic resistance of strains with keratinase activity

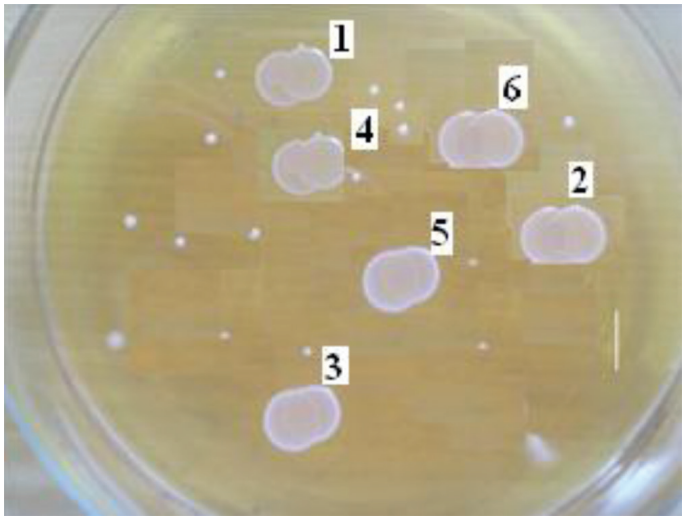

Figure 1: Evaluation of biocompatibility of strains with keratinase activity by coculture on solid medium: (1) F-626 + B-2986, (2) F-626 + F-601, (3) F-626 + S-1220, (4) B-2986 + F-601, (5) B-2986 + S-1220, (6) F-601 + S-1220

The biocompatibility of the four strains having KA was evaluated, as well as coculture conditions were selected in order to activate them.

To study the interactions between the four bacterial cultures, cocultivation method on solid medium at 30°C and pH 7.0 was used. The amount of bacterial suspension was calculated so that the final concentration of microorganisms was 1×10^3 CFU/g, 1×10^4 CFU/g, 1×10^5 CFU/g, and 1×10^6 CFU/g. The results are shown in Figure 1.

Discussion

The selection of microorganisms was based on the availability of components included in the composition of the nutrient medium, high KA, low cultivation periods, and biosynthesis of enzyme.

The highest value of biomass accumulation (Table 2) under optimal growth conditions and duration of culture was observed for 12.0 h strains *Streptomyces ornatus* S-1220 (430.0×10^3 CFU/g), *Verticillium lateritum* F-626 (342.0×10^3 CFU/g), *Bacillus licheniformis* B-2986 (280.0×10^3 CFU/g), and *Penicillium rubrum* F-601 (228.0×10^3 CFU/g). The same four strains caused maximum accumulation of the protein in the biomass (from 63.12 to 85.44%) and characterized maximum KA (from 14.6 to 32.4 U/mg) and a degree of hydrolysis of keratin (from 52.46 to 68.96%).

Based on these results, these four strains of microorganisms were selected for further studies. At the same time, the obtained results do not contradict the literature data; therefore, they are included in further studies.

The results of the studies on the microorganisms having keratinase activity show that all the above strains do not exhibit antibiotic resistance to chloramphenicol, streptomycin, tetracycline, kanamycin, and penicillin: the radius of the inhibition zone was 1.3-2.5 cm. However, each of the four strains is resistant to the antibiotic lysozyme: inhibition zone width of *Bacillus licheniformis* B-2986 is 0.2 cm, *Streptomyces ornatus* S-1220—0.3 cm, *Penicillium rubrum* F-601—0.1 cm, and *Verticillium lateritum* F-626—0.2 cm.

Thus, studies have shown that out of the 15 studied strains of microorganisms, the most active producers of enzymes with KA are the following: *Bacillus licheniformis* B-2986, *Streptomyces ornatus* S-1220, *Penicillium rubrum* F-601, *Verticillium lateritum* F-626. The values of KA of these strains, respectively, are 14.6, 32.4, 21.6, and 28.1 U/mg of protein. All the above factors favor use of industrial strains such as *Bacillus licheniformis* B-2986, *Streptomyces ornatus* S-1220, *Penicillium rubrum* F-601, and *Verticillium lateritum* F-626 for biodegradation of keratin-containing raw materials to produce high-protein feed for farm animals.

The development of a consortium consisting of these four selected strains has shown be of considerable interest.

An important feature of the strains of microorganisms is the ability to produce bacteriocins. Under the action of intracellular pheromones, enzymes cells begin to secrete molecule messengers, which accumulate in the environment, signal the growth of the bacterial population and activate a cascade of reactions that results in a stimulation of bacterial cells and the development of antimicrobial peptides. It is the spectrum of activity of bacteriocins produced determines the degree of antagonism, and the strains determine the nature of intermicrobe interactions.

In all the examined concentrations of microorganisms droplets, overlap zone is merged without the formation of clear coalesce frontiers, indicating the absence of inhibitory effect of four selected strains of microorganisms in relation to each other (Figure 1). The high degree of biocompatibility of the strains determines their appropriateness for use in the development of the technology for processing keratin-containing materials into feed for farm animals.

Conclusions

It was established that four strains show the maximum KA: *Bacillus licheniformis* B-2986 (14.6 U/mg) *Streptomyces ornatus* S-1220 (32.4 U/mg),

Penicillium rubrum F-601 (21.6 U/mg), and *Verticillium lateritum* F-626 (28.1 U/mg), which are selected for the creation of a consortium, allowing carrying out the hydrolysis of keratin-containing materials into protein supplement. The phenotypic characteristics, biochemical properties, and antibiotic resistance for four selected strains of microorganisms-destructors were studied. It was shown that all of the strains are resistant to lysozyme. The absence of inhibitory effect of the four selected strains of microorganisms—degraders in relation to each other when cocultured on solid medium at 37°C and pH 7.0—was established. The conditions for poultry industry waste destruction by the consortium consisting of four microorganisms-destructors (the ratio of strains 1: 1: 1: 1) were selected: 37°C, pH 7.5, 12.0 h duration of cultivation, the ratio of inoculum to the volume of processed raw materials 1: 5.0.

The next stage of the research is aimed at the selection of coculture conditions of the proposed combination of microorganisms with KA in order to activate them. To achieve this goal, it is planned to vary the following parameters of the hydrolysis of the poultry industry waste by consortium of microorganisms-destructor: the composition of the culture medium, temperature, pH, and the ratio of inoculum to the volume of raw material.

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