EXPERIMENTAL MODELS AND INSTRUMENTAL SURVEYS FOR RISK ASSESSMENT IN HYGIENE AND EPIDEMIOLOGY

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TOXICOLOGICAL AND HYGIENIC ASSESSMENT OF ALLERGENIC ACTIVITY AND HAZARDS CAUSED BY DRY YEAST FUNGI

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This research significance was determined by the fact that health disorders, mostly allergenic ones and immune pathologies, prevailed among workers exposed to native yeast fungi of Saccharomyces cerevisiae L153 strain and dry bakery yeast. We observed apparent shifts and imbalance between humoral and cellular immune system parameters and detected allergenic responses in a body which had occupational etiology. Our research goal was to experimentally determine sensitizing power and allergenic hazards of dry bakery, wine, and spirit yeast fungi. We developed an original technique based on oxidizing hydrolysis which we performed with an organic acid on surface β-glucoside bonds between elementary units of nitrogen-containing polysaccharide (chitin); the next stage was extraction in alkaline medium, and it allowed us to obtain extracts-allergens out of dry bakery, wine, and spirit yeast fungi with high contents of soluble protein-containing substances. It was quite sufficient for experimental modeling of their impacts on a body and detecting peculiarities of their biological effects.

We validated an alternative short-term procedure which includes unified technology aimed at reproducing and objective detection of delayed hypersensitivity during an experiment performed on white mice. This procedure allows to detect allergenic power and allergenic hazard of a biological substance using its soluble proteins-antigens.

Our experiments allowed to reveal that protein-antigen complexes contained in dry bakery, wine, and spirit yeast fungi had high sensitizing powers (allergenic powers) and belonged to the 1st allergenic hazard category (extremely dangerous occupational allergen).

We showed that bakery, wine, and spirit yeast fungi had common antigen immune determinants. It makes body poly-sensitization quite possible under inhalation exposure to them in working conditions and causes high risks of cross allergenic responses in people who contact them.

***Key words**: health disorders, inhalation exposure, yeast fungi, extracts, oxidizing hydrolysis, sensitizing power, allergenic hazard.*

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Determination of maximum allowable concentrations and control over hazardous substances contents in working area air are the most efficient measures which can prevent occupational and production-related pathologies in workers. And unfortunately, there are no existing hygienic standards for permissible contents of various microorganisms-producers and biological preparations in working area air despite their wide application in production.

First of all, we can mention such widely-spread industrial microorganisms strains as bakery, wine, and spirit yeast fungi *Saccharomyces cerevisiae*. They are applied in their natural or dry state in production of food stuffs, spirits, and wine raw materials, and a lot of workers contact them during production processes. Workers who are exposed to aerosols of native yeast fungi belonging to *Saccharomyces cerevisiae L153* and dry bakery yeast suffer from health disorders, mostly allergies and immune pathologies. Apparent shifts and imbalance between humoral and cellular parameters of the immune system occur in a body, and occupational allergic responses can also be observed [1, 2].

By now, maximum allowable concentration of yeast cells belonging to *Saccharomyces cerevisiae L153* strain in working area air has been experimentally grounded and fixed in Belarus. This maximum allowable concentration in working area air is equal to 1,000 m.cells/m^3; the strain is assigned into the 3rd hazard category and considered to be an allergen. There is also a certified procedure for measuring concentrations of such cells in working area air [3].

Another vital task here is to give grounds for maximum allowable concentration of dry bakery, wine, and spirit fungi in working area air. Dry yeast fungi which contaminate working area air belong to organic protein-containing aerosols, and methodical approaches to their hygienic standardization differ from those which are applied in standardizing viable industrial microorganisms strains. When giving experimental justification for maximum allowable concentrations of dry bakery, wine, and spirit fungi, it is necessary to determine their sensitizing power and allergenic hazard.

Our research goal was to experimentally determine sensitizing power and allergenic hazards of dry bakery, wine, and spirit yeast fungi.

Data and methods. We chose dry bakery, wine, and spirit yeast fungi as our experimental objects; these fungi were produced from a biomass of industrial yeast fungi belonging to *Saccharomyces cerevisiae* strains. Research samples were obtained from "Yeast works" LLC, located in Minsk.

We applied some techniques and methodical approaches to examining biological effects and fixing standards for organic aerosols contents in working area air; when fixing such standards, experts paid their greatest attention to proteins. Some of these approaches were developed and tested to set standards for certain organic dusts of animal, vegetative, and mixed origin [4].

To achieve our goals, first of all, we had to solve two tasks. First, to perform experimental modeling of influences exerted on a body by dry yeast cells which were practically water-insoluble, and to reveal their biological effects, mainly allergic and immune toxic ones, we should obtain soluble protein-antigen substances out of them. The task was hard to solve due to high resistance and strength of yeast fungi cell walls as they had chitin in their structure.

We tested different ways of hydrolysis of chitin and other yeast cell structures and detected optimal conditions

for the process; we also developed an original procedure for obtaining extracts-allergens with maximum possible contents of soluble protein-containing substances out of dry yeast fungi.

Techniques for experimental modeling and determining sensitization are comprehensively developed and widely used to explore industrial organic aerosols as obligate allergens and to fix hygienic standards for their contents in working area air; however, such experiments have been performed only on albino guinea pigs as these animals are the most sensitive in terms of immunologic reactivity [4]. Nowadays, it is too expensive to use these laboratory animals during experiments as they cost too much; it is even next to impossible as there isn't any facility for breeding them in Belarus. Therefore, to determine sensitizing power of dry yeast using their extracted soluble protein-antigen substances, we should solve two tasks. First, it was necessary to select and test available and objective experimental techniques for sensitization modeling and detection of delayed specific hyperimmune response to an obligate allergen; second, we had to determine criteria for assessing allergenic activity and for ranking allergenic hazards which antigen substances of dry yeast cells had.

We modified an alternative procedure (which is described below) and applied it in short-term experiments on white mice to determine sensitizing power and allergenic hazard of soluble polysaccharide-protein substances extracted from dry yeast cells. We performed our experiments on 5 groups of white mice, 12 animals in each; animals were distributed into the groups randomly in terms of their body mass (all were males, weighing 24–27 grams).

To sensitize animals and test the results, we applied obtained extracts from samples of dry bakery, wine, and spirits yeast. Sensitizing mixtures were as follows: 1:1 Freund's complete adjuvant ("Sigma") and a corresponding extract, on the basis of a standard dose per 1 animal being 0.03 cm^3 of Freund's complete adjuvant and 0.03 cm^3 of an extract, protein content being equal to 300 μg.

Laboratory animals were kept and attended to during experiments as well as taken out of them in full conformity with the requirements set forth by technical regulatory and legislative acts; all our experiments were based on international principles of biological ethics.

Research results were statistically processed with conventional techniques in STATISTICA 10 software package.

Results and discussion. To experimentally model influence exerted on a body by practically water-insoluble dry yeast cells and to reveal their biological effects, mainly allergic and immune toxic ones, it was necessary to obtain an extract out of them with maximum possible content of soluble protein-containing substances.

Literature sources have data on more than 16 different ways of how to extract antigens out of yeast and yeast-like fungi [5]. Previously, as various extraction techniques were tested, the following procedure was applied: yeast cells membranes were inactivated and partially destroyed via 4 times freezing (at –22 °C) and fast thawing; via exposure to ultrasound; via 4-day extracting out of a biomass in alkaline water-salt Coca solution under low temperature thus obtaining soluble polysaccharide-protein antigen complexes. An extract obtained out of dry bakery yeast was reaginic and antigen-isolated but it contained only 3 mg/cm^3 of protein (50000 units PNU), which was quite enough for efficient use of it as a test-allergen in laborato-

ry procedures aimed at allergy diagnostics [6], but the concentration was extremely low for any experimental modeling.

We tested another well-known technique for obtaining allergens out of coccal bacterial cells developed by V.F. Runova [6, 7]. This technique is based on extraction of protein-containing substances out of dry bacterial cells mass with 1% solution of potassium hydroxide during 1 day under room temperature with their sedimentation with 50% acetic acid solution and consequent dilution of a protein precipitate in weakly alkaline medium. This technique turned out to be efficient for obtaining diagnostic extracts-allergens out of industrial strains of Bacillus subtilis and Pseudomonas fluorescens bacteria; such extracts-allergens were proved to be highly specific, antigen-isolated, and antigen-pure. But none the less, the technique was hardly efficient for obtaining an extract out of yeast fungi.

Yeast fungi cell walls are highly resistant and rigid due to chitin which is present in their structure. Chitin is a natural polymer which comes from N-acetyl-D-glucosamine remnants bound to each other with β-(1–40)-glucoside bonds which are associated with proteins. Therefore, to obtain an extract out of dry yeast cells, we relied on a principle which underlay acid hydrolysis of glucoside bonds found in natural polymers. This principle was applied, for example, by V.V. Sokolovskiy et al. [8] to determine protein-vitamin concentrate in the environment; determination focused exactly on proteins. To perform hydrolysis of surface β-glucoside bonds between elementary units of nitrogen-containing polysaccharide of fungi cell walls, we took prepared dry yeast samples and exposed them for 5 minutes to 0.5 M water solution of trichloracetic acid under heating. Then, after fast cooling, we extracted cell suspension for 2 hours in alkaline medium (pH 8–8.4) by introducing 2H water solution of sodium hydroxide; the precipitate was then separated from the mass via centrifuging. To achieve sedimentation of protein-containing substances in the obtained supernatant, we applied cooled hydrochloric acid and centrifuging, and the precipitate was diluted in saline with 1H water solution of sodium hydroxide and pH was gradually reaching 7.2–7.4. We determined concentrations of protein-contained substances in the extracts with Lowry procedure.

The developed procedure allowed us to obtain extracts out of dry bakery, wine, and spirit yeast with high contents of soluble polysaccharide-protein substances (with protein concentrations being higher than 30.0 mg/cm^3), which was quite sufficient for experimental modeling of influences on a body and determining their biological effects. The obtained extracts were kept under –18 ^{o}C with no preservatives being added to them.

There are well-developed principles for experimental assessment of sensitizing power and allergenic hazards related to industrial chemicals and industrial biological substances [9]. These principles are based on objective quantitative criteria for taking into account a number of experimental animals with delayed hypersensitivity which was determined as per results of positive skin or intra-dermal challenge. It is important to determine significance of discrepancies between average scores of integral parameters which describe skin reactions to a challenge in a focus and a reference group of animals; this significance of discrepancies is to be determined as per Students' t criterion, or Mann-Whitney *"U"* criterion, as well as per Van der Warden *"X"* criterion. But unified techniques for sensitization and detection

of delayed hypersensitivity which are applied mostly in experiments on albino guinea pigs are costly and sometimes not available.

It makes an alternative procedure for exploring and assessing sensitizing power of chemicals which involves experiments on white mice even more attractive. According to this procedure, a mixture of a tested substance with Freund's complete adjuvant in a strictly fixed dose is applied to sensitize white mice; delayed hypersensitivity is detected on the 6th day with a skin (on an ear) or intra-dermal (in an ear or in a hind leg) challenge [10, 11]. Sensitization reproduction technique which involves Freund's complete adjuvant application on mice is based on the fact that delayed hypersensitivity is easily reproduced in them, and Freund's complete adjuvant introduction together with a tested substance enhances delayed hypersensitivity induction due to inhibition of regulatory T-lymphocytes (suppressors) subpopulations. The process is accompanied with stronger allergic reactions which allow to reveal allergenic properties of even weak chemical allergens [11].

A body reacts to a protein-containing substances (complete antigens) with a hyper-immune processes occurring in it together with mixed allergic reactions, but an immediate anaphylactic mechanism prevails here. This mechanism is promoted by antigens, namely specific immunoglobulin IgE, due to prevailing activation of helper regulation belonging to the second type of immune response. At the same time, when experts apply a mixture of an explored heteroantigen and Freund's complete adjuvant and this mixture contains tubercle bacillus antigens and is introduced intracutaneously (a "depot" is created), it stimulates a helper regulation to switch from the 2nd type immune response (Th2) to the 1st one together with development of predominantly cells-mediated mechanisms of delayed hypersensitivity [11, 12].

Given all the above-stated, we justified development of an adapted procedure for determining allergenic activity and allergenic hazard of biological substances as per their soluble protein-antigen substances during experiments performed on white mice. The procedure applied in yeast fungi examination was as follows: a mixture containing a specific extract was introduced into animals from each experimental group only subcutaneously, at the bases of their tales, in a dose equal to 0.06 cm^3; a mixture of Freund's complete adjuvant and saline was introduced in the same way and dose into animals from reference groups. On the 6th day of the experiment, we measured initial thickness of a hind leg of each animal, both in experimental and reference groups, with an electronic micrometer. After it, we introduced direct or cross provocation doses of corresponding extracts (400 μg of protein) in a volume being equal to 0.04 cm^3 into a pad (under the aponeurosis) of each measured hind leg. On the next day (24 hours after the introduction) we performed a repeat measuring of the same hind legs, calculated an absolute value of edemas in them in mm basing on a difference between thickness of white mice's legs before and after intra-dermal testing. We then transformed obtained absolute values of intra-dermal leg swelling detected in each animal into relative values of an integral parameter in scores as per a conventional scale [4]. A unified technology for conducting experiments as per this procedure allows to apply existing quantitative criteria [9] for assessing allergenic activity and detecting allergenic hazard category of protein-containing yeast fungi substances.

Application of this alternative procedure allowed us to obtain the results which are shown in the Table.

Absolute value of intra-dermal challenge was 2.4 times higher in animals from the experimental group 1 than in the reference group; a relative parameter of intra-dermal leg swelling was 8.8 times higher than in the reference group A ($t = 4.71$, $p < 0.001$). And here positive skin reactions to a challenge with an extract of dry bakery yeast with their intensity being 1–3 scores were registered in 10 out of 12 white mice from the focus group (83 % animals). So, a calculated statistical "X" criterion of a discrepancy between groups was equal to 6.77 ($p<0.01$).

Similar results were obtained during sensitization of white mice with an extract of dry spirit yeast: skin reactions to a challenge with a test dose of dry spirit fungi extract which scored 1–4 were detected in 83% animals from the experimental group 2; average group values of both absolute and relative intra-dermal leg swelling were 2.5 and 17.8 times higher correspondingly than in the reference group B ($t = 3.98$ and 4.12, $p < 0.001$). Statistical discrepancies in intra-dermal legs swelling between the focus and the reference group were authentic at $p < 0.01$ as per "X" criterion (6.93).

Frequency and intensity of intra-dermal leg swelling resulting from direct and cross challenges in white mice which were sensitized with extracts of dry bakery, wine, and spirit yeast fungi

Groups being compared	Delayed hypersensitivity as per intra-dermal leg swelling	Extracts of dry yeast fungi		
		bakery	wine	spirit
Reference group A	10^{-2} mm	7,88 ± 1,30	7,78 ± 1,10	–
	N	2/12	2/12	–
	Score	0,17 ± 0,10	0,17±0,10	–
Reference group B	10^{-2} mm	–	–	7,76±1,16
	N	–	–	1/12
	Score	–	–	0,08 ± 0,08
1 experimental group, bakery	10^{-2} mm	19,0 ± 2,30**	–	15,5 ± 2,24*
	N	10/12	–	9/12
	Score	1,50 ± 0,30**[1)]	–	1,08 ± 0,22**[1)]
22 experimental group, spirit	10^{-2} mm	–	13,7 ± 1,70*	19,6 ± 2,74**
	N	–	9/12	10/12
	Score	–	1,00 ± 0,20*[1)]	1,42±0,31**[1)]
33 experimental group, wine	10^{-2} mm	18,2 ± 2,60*	23,6 ± 3,40**	–
	N	9/11	9/11	–
	Score	1,18 ± 0,30*[1)]	1,64 ± 0,30**[1)]	–

Note: * – authentic discrepancies from the reference group at $p<0.01$ as per t criterion,
** – authentic discrepancies from the reference group at $p<0,001$ as per t criterion
1) – authentic discrepancies from the reference group at $p<0.01$ as per "X" criterion,
N: numerator is a number of animals with positive intra-dermal leg swelling parameters, denominator is a total number of animals in a group.

A bit elevated absolute and relative intra-dermal leg swelling was detected in white mice sensitized with dry wine yeast extract; average groups parameters in the 3rd experimental group were 3 and 9.6 times higher correspondingly than in the reference group A (t=3.82 and 4.30, p<0.001). Frequency of apparent skin reactions (1-3 scores) in the experimental white mice from the 3rd group amounted to 81.8%, and the discrepancy between relative intra-dermal leg swelling in the experimental and reference group (7.05) was authentic at p<0.01.

So, extracts-allergens obtained from dry bakery, wine, and spirit fungi sensitized more than 75% animals from the experimental groups under standard experimental conditions; average mean values of integral intra-dermal leg swelling parameter were authentically different in animals from the experimental and reference groups as per "X" criterion at p<0.01. As per classification criteria [9], these data allow us to rank dry bakery, wine, and spirit yeast fungi as having great sensitizing power (allergenic activity) and to assign them into the 1st allergenic hazard category (extremely dangerous industrial allergen).

We should also note that that there was high frequency and intensity of skin reactions in sensitized animals from the experimental groups which occurred as a response to a cross testing with test-allergens of various yeast fungi strains; these reactions were authentically significantly stronger than in white mice from the corresponding reference groups (p < 0.01 as per t and "X" criteria). Although both absolute and relative intra-dermal leg swelling which occurred in animals from the experimental groups as a response to cross test-doses of dry bakery, wine, and spirit yeast was lower than that occurring as a response to specific extracts-allergens, but still it didn't differ statistically significantly in terms of its intensity and frequency. Thus, for example, positive skin reactions to a test allergen of dry spirit fungi extract were detected in 9 out of 12 white mice from the 1st experimental group which were sensitized with dry bakery yeast extract and their relative intra-dermal leg swelling was equal to 1.08±0.22 scores; and in case of a response to a specific test-allergen of dry bakery yeast extract it was equal to 1.50±0.30 scores (t=1.19, p>0.05). Cross reactions occurring due to application of various fungi allergens, even to a fungi belonging to different strains, have been known for some time already, and antigen similarity between fungi belonging to the same species is even more substantial [5, pages 76–79]. Thus, when guinea pigs were sensitized with Candida albicans cells, experts detected high frequency and intensity of cross allergic reactions in them to mold fungi antigens and even to penicillin which was their producer [13, pages 87–89].

So, occurrence of common antigen immune determinants in dry bakery, wine, and spirit yeast fungi makes poly-sensitization quite possible under inhalation exposure to them and causes high risks of cross allergenic reactions in people who contact them.

This fact is important as it helps to justify the same maximum allowable concentration in working area air for all these fungi and to apply a standardized strain of dry yeast cells as a reference allergen.

Conclusion. The results of the performed experimental research allow us to make the following conclusions:

1. We developed an original procedure based on oxidizing hydrolysis performed with organic acid on surface β-glucoside bonds between elementary units of nitro-

gen-containing polysaccharide (chitin) of fungi cell walls with consequent extracting in alkaline medium. It allowed us to obtain extract-allergens out of dry bakery, wine, and spirit yeast fungi with high contents of soluble protein-containing substances which was sufficient for experimental modeling of their influences on a body and determining their biological effects.

2. We validated a short-term alternative procedure which includes a unified technology for reproducing and objective detection of delayed hypersensitivity during an experiment performed on white mice. This procedure allows to determine allergenic activity and allergenic hazard category of biological substances focusing on their soluble protein-antigen components.

3. We experimentally revealed that protein-antigen complexes contained in dry bakery, wine, and spirit fungi have substantial sensitizing power (allergenic activity) and are assigned into the 1st allergenic hazard category (extremely dangerous industrial allergen).

4. Bakery, wine, and spirit yeast fungi have common antigen immune determinants and it makes body polysensitization highly possible under inhalation exposure to them at a work place and causes high risks of cross allergenic reactions in people who contact them.

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