Effect of nickel oxide nanoparticles on protease activity in brewing industry

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Abstract. Intensive use of nanoparticles on an industrial scale leads to an increase in their content in the environment. This increases the risks of nano-sized objects entering the technological chains of the processing industries of the agro-industrial complex, in particular, brewing. The paper presents the results of the studies of the effect of nickel oxide nanoparticles used in various industries on the activity of the proteolytic type of enzyme preparation Neutrase 1.5MG, as well as on the results of laboratory mashing of light barley malt. The effect of different concentrations of NiO nanoparticles on the accumulation of low molecular weight nitrogenous substances during gelatin hydrolysis in model media was determined. It was found that if the content of nanoparticles exceeds 0.25 mg/cm$^3$, the proteolytic capacity of the enzyme preparation is reduced up to 70% compared to the control at the concentration of nickel oxide nanopreparation of 2.0 mg/cm$^3$. The experiments showed that an increase in the duration of contact between nanoparticles and proteases of the enzyme preparation in the reaction medium did not lead to an increase in the inhibitory effect of the nano-sized NiO. The laboratory mashing revealed more pronounced negative effect of nickel oxide nanoparticles on the accumulation of low-molecular nitrogen compounds. It is determined that in the presence of NiO nanopreparation, the hydrolysis efficiency of the starchy components of light barley malt is reduced. As a result, according to a number of indicators (concentration of amine nitrogen, reducing substances), the first wort obtained by mashing in the presence of nickel oxide nanoparticles at both lower (0.25 mg/cm$^3$) and higher (2.0 mg/cm$^3$) concentrations is inferior to samples obtained in the absence of nano-sized particles. Based on the above data, it is concluded that the presence of NiO nanoparticles in brewing environments is undesirable.

1. Introduction
Receiving, properties studying and applying the nano-sized objects (nanoparticles and nanopreparations) and materials on their basis on an industrial scale is one of the most intensively developing directions of science and technology of the last decades. It was proved that such objects have properties that are uncharacteristic for those having a micro-, especially macro-size [1], and their application in various industries allows solving a wide range of problems [2–5]. The most typical areas today are the following:
- medicine: diagnosis of diseases, “point” delivery of drugs and pharmacological preparations, artificial retina;
- construction: nanodetectors of building structures and building materials with improved properties;
- energy: improving the efficiency of solar panels;

- instrumentation and automobile manufacturing: single-electronic devices, chargers of a new type, air cleaners, structural materials of increased strength;
- military products.

It should be taken into consideration that one of the most characteristic properties of nanoparticles is their highest migratory ability, which in particular allows them easily overcoming biological barriers, including placental and haematoencephalic [6]. Thus, it is inevitable that nanoparticles enter the environment, especially large-scale thus causing a larger the use of nanoparticles in production practice. As a result, there are the risks of nanoparticles entering in the composition of raw materials in those industries in which nano-size object’s use and presence are not expected. Separately, it is worth mentioning innovative techniques involving the introduction of certain nanoparticles (for example, silver) having the highest microbicidal effect into the packaging of food products, in particular food polymer films, as a result of which the migration of nanoparticles into the product will take place directly, without “intermediaries”. A similar situation may occur in the use of textile fiber and fur products which surface is modified with inorganic nanoparticles, for example silver, to impart stable antibacterial properties and/or improve quality, as well as in the introduction of nanoparticles into plant growth media.

At the same time, it is necessary to take into account the increasing volume of publications [7, 8], which provide experimental data showing the pronounced negative effect of nanoparticles on the characteristics of objects of different levels of organization, as well as on processes taking place in them or with their participation. The identified negative impact may not be selective, but rather universal. Obviously, due to this, in recent years a whole scientific direction has been formed – “nanotoxicology” [9], the purpose of which is to assess the impact of nano-sized objects, primarily on human health, as well as on other biological objects.

In our opinion, this made it expedient to study the impact of nanoparticles of various types on raw materials, aids and brewing processes. This is due to the fact that both technology and the results of this production process significantly depend on biological (brewing barley, yeast, cells of spoilage microorganisms) and biochemical (enzymes of raw materials and enzyme preparations, if used) objects. This view is confirmed by the studies previously conducted at the Moscow State University of Food Production. Thus, they describe the presence of a pronounced, in many cases, negative effect of certain types of nanoparticles (silver, copper, iron, zinc oxides, multilayer carbon nanotubes) on the development of yeast populations, barley germination, activity of enzymes and enzyme preparations, flow and results of the mashing in brewing [10, 11]. The direction and intensity of such influence depended on a number of factors, first of all, on the type of nanoparticles, their content in the medium, the type of object affected, the duration of its contact with nano-sized objects. Due to this, it was decided to expand the range of nanoparticles, the role of which in brewing is expedient to study.

Nickel oxide nanoparticles were chosen for this purpose. Nanoparticles/nanopreparations of nickel oxide are produced in a significant amount, about 15,000 tons annually. They are widely used on an industrial scale, for example, in electrical engineering, nanooptics, biodiagnostics (in the manufacture of biochemical sensors), chemical and metallurgical industry, in the production of solar cells/elements. This allows improving the quality of finished products or reducing the cost of production.

Metal oxides are capable of undergoing a number of chemical reactions. However, nano-sized particles acquire special properties due to the fact that the “continuous” density of states existing on the macro scale is replaced by discrete levels, with distances between them dependent on particle sizes. On such a scale, the material no longer exhibits the physical properties characteristic of the same substance in the macro state or exhibits them in a modified form. In particular, it acquires increased toxicity to biological objects [12]. Therefore, we considered it appropriate to study the effect of the nickel oxide nanoparticle on the activity of proteolytic enzymes.

An enzyme preparation of microbial origin “Neutrase 1.5MG” was chosen as the object of exposure to NiO nanoparticles. This choice was caused by the fact that this enzyme preparation is quite widely used in a number of branches of food production. In a technologically significant amount,
it contains almost only proteolytic enzymes. Therefore, it was used to evaluate the effect of nickel oxide nanoparticles on the activity of microbial proteases.

2. Materials and methods

The subject of research was the enzyme preparation Neutrase 1.5MG – a preparation of neutral protease, producer – genetically unmodified strain Bacillus amyloliquefaciens [13]; characterized by brown color, acts as a loose microgranulate, does not spray. The average particle size is 300 microns. Activity – 1.5 AU/g, AU = Anson activity units. Optimum operating conditions: temperature – up to 70 °C, pH – 5.5-7.5. It catalyzes the hydrolytic cleavage of protein substances, promotes the formation of low molecular weight nitrogenous substances.

NiO nanopreparation manufactured by Plasmeterm, Moscow, RF: particle size range – 40-130 nm, nickel oxide content – 99.6%, color – black, particle shape – spherical [14].

Wort extract content was determined by the pycnometric method.

The duration of mash saccharification was determined by the time until the starch-iodine sample did not change its color.

The concentration of reducing agents was determined by the DNSA method.

The concentration of amine nitrogen was determined by the number of carboxyl groups in the aqueous alcohol solutions.

All determinations were carried out in three iterations, the figures and tables show the mean values.

3. Dependence of proteolytic activity of microbial enzyme preparation on the presence of nickel oxide nanoparticles in reaction media

At the first stage, it was decided to establish the presence or absence of an influence of NiO nanoparticles on the proteolytic activity of the enzyme preparation Neutrase 1.5MG used as an object of influence. To do this, a series of experiments were carried out, the conditions of which were identical, with the exception of the content of nickel oxide nanoparticles in the reaction medium, which varied in the range 0.1-2.0 mg/cm³. In general, the conditions of each individual experiment were as follows: a 5% solution of food gelatin was used as a substrate, to 10 cm³ of which 2 cm³ of an enzyme preparation solution with the concentration of 2 mg/cm³ or distilled water was added, depending on the variant (control, comparison or test). Besides, in one variant, prior to addition of the enzyme preparation solution to the substrate, the nanopreparation of nickel oxide was added in a manner that provided the content of nanoparticles for a specific experiment. In total, in each experiment of the first series, three variants of the following composition were set and analyzed:

- gelatin solution and distilled water (to control change of amine nitrogen content in substrate solution without proteases and/or nanoparticles);
- gelatin solution and enzyme preparation solution (in fact, control to evaluate the efficiency of proteases of the enzyme preparation);
- gelatin solution, enzyme preparation solution and a certain amount of nanopreparation (experimental version).

Immediately after the variants were formed, samples of 1 cm³ were taken from each of them to determine the initial concentration of amine nitrogen by the number of carboxyl groups in aqueous alcohol solutions.

All variants were then thermostatted for 60 minutes at 40 °C for enzymatic hydrolysis in experiments and control variants. Thereafter, the sampling was repeated to determine amine nitrogen using the above method.

Protease activity (PA) was evaluated by increasing the concentration of low molecular weight nitrogenous substances during the enzymatic reaction, minus the amount by which the concentration of amine nitrogen in the variant without the enzyme preparation solution changed under enzymatic reaction conditions.

For the convenience of comparing the results of certain experiments of the series under discussion, the increase in the concentration of low molecular weight nitrogen-containing compounds in the
control version of each individual experiment (not containing nanoparticles) was taken as 100% (the data in the summary graph are not shown), the same indicator of the test version was also expressed as a percentage. The results of the experiments of the entire series are shown in Figure 1.

![Graph showing the dependence of PA enzyme preparation Neutrase 1.5MG on the content of nickel oxide nanoparticles in reaction medium.](image1)

**Figure 1.** Dependence of PA enzyme preparation Neutrase 1.5MG on the content of nickel oxide nanoparticles in reaction medium.

In our opinion, based on the above data it can be concluded that under experimental conditions nickel oxide nanoparticles showed a significant effect on the activity of proteolytic enzymes of microbial origin. So, at the concentrations of nanoparticles from the range of 0.1 - 0.25 mg/cm\(^3\), nanoparticles did not change the PA of the enzyme preparation – small deviations (-5.3 ± 1.9%) of the same indicator of the control variant, in our opinion, are caused by determination errors. However, further increase in the content of nanoparticles led to gradual decrease of the values of a controlled indicator: by 24% at 0.5 mg/cm\(^3\), by 37% at 1.0 mg/cm\(^3\) and by 68% at 2.0 mg/cm\(^3\) of NiO nanoparticles.

Clearly, the likelihood of nickel oxide nanoparticles being present in real production environments now seems highly unlikely. However, the results given, from our point of view, allow concluding that nanoparticles of the used type may have a negative effect on biocatalysts, at least of microbial origin, especially at their increased content in reaction media, or, probably in the case of nanoparticles of several types, even with low content of each of the varieties.

In the next phase of our work, the experiments were aimed at verifying earlier findings on the direction and intensity of the effect of nickel oxide nanoparticles on microbial protease activity, as well as assessing the role of the contact duration of enzymes and nanoparticles in the reaction medium, since earlier studies established a significant impact of this factor on controlled indicators of objects, in particular, the activity of enzymes of various origins and types of action.

The conditions of these experiments and the processing method of experimental data were identical to those described above, except that the duration of the enzymatic reaction, and therefore, the contact time of NiO nanoparticles and proteases was increased to 3 hours, and the sampling from all three variants were taken every 60 minutes.

![Graph showing accumulation of amine nitrogen in the presence of NiO nanoparticles (0.25 mg/cm\(^3\)) in the reaction medium.](image2)

**Figure 2.** Accumulation of amine nitrogen in the presence of NiO nanoparticles (0.25 mg/cm\(^3\)) in the reaction medium.
Figure 3. Accumulation of amine nitrogen in the presence of NiO nanoparticles (2.00 mg/cm$^3$) in the reaction medium.

Besides, Figures 2 and 3 show amine nitrogen concentrations in natural terms (mg/100 cm$^3$) rather than as a percentage of control. In experiments, nanoparticles were introduced into the reaction medium at concentrations at which the largest increase (0.25 mg/cm$^3$) and decrease (2.00 mg/cm$^3$) of the enzyme preparation PA were recorded in the previous series of experiments.

From our point of view, Fig. 2 and 3 make it possible to conclude that at the content of NiO of nanoparticles equal 0.25 mg/cm$^3$, the activity of microbial proteases almost does not change in comparison with the control, whereas the increase of nanoparticle concentration to 2.00 mg/cm$^3$ resulted in a significant drop in the PA of the enzyme preparation, although not as significant as was found under similar conditions in the previous series of experiments (after 60 minutes of the reaction).

4. Effect of nickel oxide nanoparticles on the brewing mash preparation

The conditions of hydrolytic processes on an industrial scale differ significantly from those observed in model media. Therefore, at the final stage of the study it was decided to define the influence of nanodimensional particles of nickel oxide on the activity of proteases of the enzymatic preparation Neutrase 1.5MG in the conditions close to the industrial ones, for which laboratory mashing of variants containing and not containing both microbial proteases, and NiO nanoparticles was carried out.

The conditions of the first experiment of the series under discussion were as follows:
- mash composition for each of the variants – 20 g of malt charge (light barley malt, crushed) and 80 cm$^3$ of tap water;
- nickel oxide nanoparticles and/or enzymatic preparation were added to the water of respective variants prior to malt charge;
- mashing was carried out with three pauses: at 50-52 °C, at 60-63 °C and at 70-72 °C, the duration of each – 20 minutes;
- samples for saccharification completeness were taken after the mash reached a temperature of 70 °C, every 5 minutes throughout the saccharification pause;
- at the end of the saccharification pause, mash, regardless of the color of the last iodine-starch sample, was transferred to filtration through the spent grains settling, returning the filtrate to the filter layer of the spent grains until the filtrate achieved visual transparency;
- filtration was carried out until the cracking of the spent grains settling.

The following variants were set and processed:
1. without nickel oxide nanoparticles and enzyme preparation (control);
2 – with enzyme preparation Neutrase 1.5MG at a dosage of 0.5% to a malt charge (a comparison variant that allows assessing the results of the joint action of proteases of plant and microbial origin under experimental conditions);

3 – with enzyme preparation Neutrase 1.5MG at a dosage of 0.5% to a malt charge and the nanopreparation of nickel oxide in the amount providing their content equal to 0.25 mg/cm$^3$ (experimental variant, which allows determining the influence of NiO nanoparticles in specified concentration on the hydrolysis of protein substances of light barley malt).

In each sample of the first wort obtained after mash filtration, the extractability, the content of reducing substances by the DNSA method and amine nitrogen by the number of carboxyl groups in aqueous alcohol solutions were determined (Table 1).

**Table 1.** Characteristics of the first wort obtained in the presence of NiO nanoparticles (0.25 mg/cm$^3$).

| Variant                    | Duration of saccharification, min | Extract content, % | Concentration of reducing substances, mg/cm$^3$ | Concentration of amine nitrogen, mg/100 cm$^3$ |
|---------------------------|----------------------------------|--------------------|-----------------------------------------------|-----------------------------------------------|
| control                   | 20                               | 12.86 ± 0.43       | 53.0 ± 1.8                                    | 43.40 ± 1.6                                   |
| comparison (+ EP Neutrase 1.5MG) | 15                               | 12.43 ± 0.29       | 60.0 ± 1.3                                    | 50.84 ± 1.4                                   |
| test (+EP Neutrase 1.5MG + NiO nanopreparation) | 20                               | 12.61 ± 0.41       | 48.0 ± 1.4                                    | 42.60 ± 1.1                                   |

A number of conclusions can be drawn from Table 1:
- unlike the hydrolysis of gelatin in the model medium under the action of enzyme preparation Neutrase 1.5MG, the presence of nickel oxide nanoparticles in the mash at the concentration of 0.25 mg/cm$^3$ negatively affected the accumulation of low molecular weight nitrogen compounds; the reduction of their concentration in the first wort of the test version was more than 16%;
- slight difference in the concentration of amine nitrogen in the first wort of the test and control variants suggests that the presence of nanoparticles in the used concentration adversely affected the proteases of the enzyme preparation more than malt enzymes of the same type of action;
- presence of nanoparticles also led to a decrease in starch hydrolysis, as evidenced by both the duration of saccharification and the concentrations of reducing agent; this can be caused by both direct inhibition of malt amyloses and the negative effect of the nanopreparation on microbial and malt proteases, the manifestation of which increases the effectiveness of starch-reducing enzymes of the mash.

Thus, it was found that even if the content of NiO nanoparticles in the mash is 0.25 mg/cm$^3$, the indicators of the first wort are inferior to the same characteristics of samples obtained using the enzyme preparation Neutrase 1.5MG and without it. Therefore, the negative effect of nanoparticles in the mash on the hydrolysis of protein substances is more pronounced than in the previously used model medium.

In the conclusion of our studies, another experiment was carried out, the conditions of which completely coincided with the conditions of the previous one, except that nickel oxide nanoparticles were introduced into the experimental variant of the mash in such an amount that their content was equal to 2.0 mg/cm$^3$. The first series of experiments at such content of nanodimensional NiO particles recorded the essential inhibition of microbial proteases at the hydrolysis of gelatin in the model environment. The results of the experiment are shown in Table 2.
Table 2. Characteristics of the first wort obtained in the presence of NiO nanoparticles (2.00 mg/cm$^3$).

| Variant                        | Duration of saccharification, min | Extract content, % | Concentration of reducing substances, mg/cm$^3$ | Concentration of amine nitrogen, mg/100 cm$^3$ |
|--------------------------------|----------------------------------|--------------------|-----------------------------------------------|---------------------------------------------|
| control                        | 20                               | 12.56 ± 0.38       | 49.0 ± 1.4                                    | 40.43 ± 1.7                                 |
| comparison (+ EP Neutrase 1.5MG) | 15                               | 12.21 ± 0.40       | 56.0 ± 1.1                                    | 49.45 ± 1.5                                 |
| test (+EP Neutrase 1.5MG + NiO nanopreparation) | 30                               | 12.33 ± 0.22       | 44.0 ± 1.2                                    | 18.02 ± 1.0                                 |

Table 2 made it possible to conclude the following:
- the inhibitory effect of the used content of nanoparticles in the mash on the hydrolysis of barley malt proteins was very pronounced: the decrease in the concentration of amine nitrogen in the test variant was 63% compared to the comparison variant and 55% compared to the control; this inactivating effect is close to that observed under conditions of gelatin hydrolysis in the model medium;
- under the conditions of the experiment, most likely that NiO nanoparticles reduced the activity of proteases of both vegetative (malt) and microbial (enzyme) origin;
- with the content of nickel oxide nanoparticles equal to 2.0 mg/cm$^3$, under experimental conditions, significant inhibition of amylases was recorded, which led to an increase in the duration of saccharification (1.5-2 times) and the decrease in the concentration of reducing substances in the first wort (10-21%) compared to the control and comparison variants;
- the earlier conclusion that the concentration of nickel oxide nanopreparation is a significant factor determining the intensity of negative effects of nanoparticles on the microbial enzyme preparation PA and probably on hydrolytic enzymes of other origin and type of action was confirmed.

5. Conclusion
Based on all the data presented, we believe that the potential presence of nickel oxide nanoparticles in the process media, at least at the concentrations of 0.25 mg/cm$^3$ or more, may negatively affect the brewing processes based on proteolytic enzymes. This should be taken into account by technologists, and the presence of NiO nanoparticles should be compensated by changing the parameters of individual stages (increasing the duration, increasing the dosages of enzyme preparations, etc.) or will require the development and use of techniques for removing nano-sized particles/reducing their content to a safe level.

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