Dry Heat Inactivation of *Bacillus cereus* in Rice

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Abstract

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The aim of this work was to validate the method of decontamination of rice at the temperature of 120°C (determined as optimal in previous experiments). *Bacillus cereus* was selected as the marker micro-organism for the monitoring of decontamination. The spores of *Bacillus cereus* are moderately heat resistant. In order to show the efficacy of our decontamination process, we artificially contaminated the rice under study with *B. cereus*. Decontamination was carried out in a homogenising steriliser about 20 h after contamination. The sample was first heated to 90°C and held at this temperature for 70 minutes. Then the temperature was increased to 120°C and held for 3 hours. Five samples were taken for microbiological analyses as follows: before the experiment, on reaching 120°C, and then after 1, 2, and 3 h of decontamination. Decontamination of rice from vegetative forms and spores of *B. cereus* present at the level of 400 CFU/ml was effected by heating to 120°C and holding for 1 hour.

Keywords: rice; sterilisation; dry heat; *Bacillus cereus*

Bacillus cereus is known as the cause of food poisoning outbreaks in which the vehicle food is often rice (Krämer 1992; Baumgart 1994). Kleer *et al*. (2001) showed that *B. cereus* caused food illnesses in the German army in the years 1985–2000 in 30% of individual cases and in 42% of outbreaks. The rice concerned is cooked as part of a meal in restaurants or catering establishments. The illness is intoxication (formerly called food poisoning) because vegetative cells of certain serotypes of *B. cereus* create as its metabolites one of two toxins directly on the food substrate. It is either the toxin causing diarrhoea or the toxin causing vomiting. The latter one occurs almost exclusively after contaminated rice or rice meals consumption. Epidemiological analyses have shown that for the intoxication to occur (by either type of toxin) it is necessary for *B. cereus* to grow intensively in the food and to reach the concentration of 10^5 to 10^6 per gram.

It is usually presumed that the presence of *B. cereus* in the cooked meal is the consequence of secondary contamination, i.e. while vegetative cells and spores were destroyed, spores naturally occurring, in the air fall subsequently into the food. These spores then germinate into vegetative forms which can multiply, and if their concentration reaches the critical level, the meal can cause intoxication.

It is less well known that the spores of *B. cereus* are moderately heat resistant and that boiling in aqueous environment kills them only after a longer time than is the time of boiling necessary for reach-

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ing the culinary acceptable state. Decimal reduction times D_{95} ranged from 1.5 to 36.2 min in distilled water. Considerable variations were observed between different strains (Lake *et al*. 2004).

Azanza and Centeno (2004) determined the decimal inactivation times for Philippine strain 1061 spores of *B. cereus*. They found D_{100} equal to 5 minutes. The authors found in the literature that the known initial maximum concentration of spores in rice was 10^5/g. The total heat inactivation time was therefore determined as 25 min at 100°C. After this time and temperature history, the rice is usually already overcooked.

This is why the spores, which were present in uncooked rice and survived the usual heat treatment, can under some conditions survive and germinate in the cooked rice into vegetative forms and subsequently multiply. Germination of spores (e.g. in cooked rice) requires temperature in the range from 5°C to 50°C. The temperature for the growth of vegetative forms is 15–50°C, with the optimum growth at about 30–37°C (Lake *et al*. 2004).

Dufrenne *et al. (*1994) determined the minimum temperature for the growth, the generation time, and the decimal inactivation time at 90°C for 30 strains of *B. cereus*. The minimum temperature found was around 7°C and the strain showed the generation time of about 8 hours. This shows the dangers of *B. cereus.*

In this respect, it is important to know the temperature range of the greatest production of toxin. Szabo *et al*. (1991) addressed this and determined that the optimum range for the formation of the toxin of *B. cereus* is from 25°C to 30°C. The minimum recorded temperature of the toxin formation was 15°C.

As concerns the psychrotrophic forms of *B. cereus*, the minimum growth temperature is only 5°C. The generation time (e.g. in cooked rice) at 30°C can be as short as 26 min, the number of cells can therefore double every half an hour and multiplication that proceeds according to the geometric sequence can, under such conditions of keeping the cooked meal, reach the critical concentration. It is clear that keeping cooked meals at room temperature for a longer time is highly hazardous and totally unsuitable from the hygiene point of view.

The presence of *B. cereus* spores in rice as a raw material cannot be excluded under usual conditions. As a highly resistant form of this microorganism, the spores occur especially in soil from which they get into the air, water, and numerous foods. Because of their high resistance, it would be desirable to eliminate them from raw rice. Sarrias *et al.* (2003) attempted this by irradiating rice in distilled water with electrons and determined the doses necessary for the inactivation of micro-organisms including the spores of *B. cereus*. Grande *et al*. (2006) applied enterocin AS-48 and developed a very effective procedure of inactivation of live cells and spores of *B. cereus* that suppressed the toxin production in rice and rice pudding (mash).

The aim of this work was to test the physical method of microbial decontamination of rice by the so called dry heat during mixing in a homogenising steriliser.

MATERIAL AND METHODS

Procedure for rice contamination. A homogenising steriliser was used in the experiments. It consists of an electrically heated horizontal cylinder. Inside the cylinder is a rotor with mixing arms and wipers that perfectly wipe the walls of the cylinder and ensure the mixing of the content. The detailed description of the installation is, for example, in Kýhos *et al.* (2004), or in Landfeld *et al*. (2006). The charge of rice grains introduced into the steriliser was 9 kg. The rice was tempered at 30°C for 1 hour. After this, the rice was contaminated by the prepared inoculum of *B. cereus* in physiological solution. The total volume of the inoculum was 1000 ml and it was poured into the rice in six small doses step by step.

The surface of the rice grains was coated with the inoculum directly from the transfer vessels six times in total. After each application, the rice was mixed in the homogenising steriliser for 2 minutes. The total time of contamination was 15 minutes. The rice was wet and sticking to the internal mechanical elements. The steriliser was left open and a stream of air was induced by the temperature difference between the interior and the suction tube (chimney) heated to 65°C. The temperature of 30°C was maintained in the steriliser for 4 h during constant homogenisation with 44 revolutions of the rotor per minute. The rice stopped sticking and started to flow and mix after 30 minutes. After 4 h, the apparatus was switched off and the rice was allowed to cool spontaneously to the temperature of 20°C.

Procedure for the decontamination of rice. The following day (20.5 h after contamination),

the thermal decontamination commenced. Before the experiment, after a short homogenisation, a sample of rice was taken (sample N_1). After this, the heat source was switched on and set at 90°C. This temperature stabilised after 60 min and was held for another 70 minutes. The apparatus was ventilated again by the temperature difference in the apparatus and the exhausting tube (chimney) was held at 120°C. Subsequently, the temperature of the apparatus was increased to 120°C. This increase was achieved in 45 minutes. When the temperature of 120°C was reached, sample N_2 was taken, after which the decontamination time itself followed. Samples N_3, N_4, and N_5 were taken at 1-h intervals. The sample for determining dry matter was taken at the same time as sample N_5. This concluded the experiment. The temperature of the rice grains was measured directly on the heat transfer surface and in the centre of the apparatus at the shaft.

The sampled rice was poured into sterile plastic bags that were immediately sealed by welding and chilled to about 5°C. The samples were kept in a chilled thermally insulated container in which they were transported immediately after the experiment to the laboratory of the State Health Institute for microbiological analysis.

Description of the culture used. The culture *Bacillus cereus* CCM 2010 used for the experiments is a culture with defined properties and is equivalent to the culture from the American Collection of Cultures with the designation ATCC 14579. Czech collection of micro-organisms (CCM) supplies the culture in the form of gelatine disks in sterile bottles in the quantities of 20 pieces. Revitalised working culture was prepared from each disk by a procedure recommended by CCM on the so-called slanted agar (SA). The revitalisation took place at 37°C, with the total duration for 24 hours.

Preparation of the working culture. The standard liquid medium BHIB (Brain Heart Infusion Broth) divided into aliquots of 9 ml in test tubes was inoculated with the revitalised culture from the SA. With a view to standardise the inoculum, the culture was transferred with a full loop calibrated for the volume of 1 µl; this was done three times, each time using a new loop. The inoculated medium was incubated at 30°C for 18 h in order to achieve the so called early stationary phase of growth that is normally recommended and used for the experiments investigating lethal and sub-lethal actions on bacteria, e.g. Schlemmerová (1987).

Determining the counts of *Bacillus cereus.* Vegetative cells of *B. cereus* were quantified using a special growth medium designated for this purpose according to the standard ČSN EN ISO 7932. The technique of inoculation consists of spreading the inoculum in the volume of 0.2 ml on the surface of MYP (Mannitol-egg yolk-polymyxin) agar plates, each time in parallel. The conditions during incubation complied with the same standard: 18–48 h at 30°C. For the first count, the duration is 18 to 24 h, and only when the colonies are not clearly visible, the incubation is extended to the total of 48 hours. The selected culture of *B. cereus* CCM 2010 grew very well in typical colonies already after 18 h; this is why we chose this duration for all experiments.

The spores of *B. cereus* were quantitatively determined according to ČSN 56 0100, article 87. The chosen dilutions of the working culture were subjected to heating in a water bath previously tempered to 70°C for 15 minutes. This action leads to the inactivation of the present vegetative cells while the spores survive. Using these treated dilutions, we inoculated the quantities of 0.2 ml (always in parallel) onto the surface of non-selective agar plates (PCA – Plate Count Agar), spreading the inoculum on the surface in the usual way. The conditions of incubation according to the same standard, i.e. 3 days at 30°C, were modified by extending the incubation time to the total of 4 days. The method does not detect the number of all sporulating cells but only the number of viable spores that are able to germinate and form colonies on PCA.

Confirmation of *B. cereus.* During the analysis of intentionally contaminated rice, it was also necessary to include the differentiation of species that we introduced by inoculation from the species that were already naturally present in the raw rice. The confirmation of *B. cereus* was carried out according to ČSN EN ISO 7932. This is important especially for the differentiation of colonies grown from spores on the non-selective medium PCA where the differentiation of *B. cereus* by using the signs of non-utilisation of manitol and the formation of lecitinase logically does not apply.

Preparation of inocula of predetermined density. In order to ensure a favourable outcome of the final experiment, i.e. to achieve a good comparability of the states before and after the heat treatment, it was necessary to carry out several preliminary experiments. These were directed towards the estimation of the density of vegetative cells of *B. cereus* in the working culture, the selec-

tion of the dilution ratios of the working culture for the contamination of rice, and another important factor – the ratio of the volume of the inoculum to the mass of raw rice. This is important with respect to the soaking ability of rice that leads to the reduction of the detectable microbial cells in the classical homogenate of the sample as it is prepared for laboratory microbiological analysis according to ČSN ISO 7218.

For the contamination of rice, we selected the dilution of the working culture of 10^{-2} and expected the density of vegetative cells of *B. cereus* to be of the order of 10^5. This was confirmed in the final experiment where the value of N_0 was 6.0×10^5/ml. It was necessary to prepare unusually large volume of inoculum for the laboratory conditions, specifically 1000 ml with which 9 kg of rice was intentionally contaminated. This ratio was also chosen according to the preliminary experiments. The working culture of the volume of 10 ml was introduced into 990 ml of standard diluting solution according to ČSN EN 6888-1 (physiological solution with the addition of peptone), which corresponded to the dilution of the working culture of 10^{-2}.

Microbiological analysis of samples of intentionally contaminated rice. The samples taken under aseptic conditions into five previously labelled sterile sacs for laboratory homogeniser STOMACHER were investigated in the way usual for foodstuffs. Into another sterile sac of mass 10 g, we added 90 ml of standard diluting solution and homogenised the contents for 2 minutes. Immediately after this, we prepared decimal dilutions and inoculated selected dilutions onto the medium MYP. The dilutions were subjected to heating (inactivation) and inoculated onto PCA for the investigation of the number of viable spores.

Method of calculation. For the calculation of the numbers of vegetative cells of *B. cereus* and its confirmed spores before and after the heat treatment (N_0 and appropriate N_1 up to N_5), we proceeded according to the version of ČSN ISO 7218 which includes the amendment Amd. 1:2001 and presents a general equation for calculating the weighted average:

$$N = \frac{\Sigma C}{V \times [n_1 + (0.1 \times n_2)] \times d}$$

where:

ΣC – sum of the number of confirmed colonies from all plates selected for the calculation from two consecutive dilutions where at least one plate contains at least 15 colonies

V – volume of inoculum in ml, inoculated onto each plate

n_1 – number of plates selected for the calculation from the 1st selected dilution

n_2 – number of plates selected for the calculation from the 2nd selected dilution

d – dilution factor corresponding to the 1st dilution selected for the calculation

Moisture and water activity measurements. Moisture content of rice samples was measured by drying in hot air at the temperature of 105°C for 24 hours. Hot air steriliser HS62A (Chirana, Czech Republic) was used for drying the samples.

Samples of rice with different moisture contents for the prediction of desorption isotherm were prepared by slow drying of pre-wetted rice. Water activity of these samples was measured by the water activity sensor, Rotronic AM3 (Switzerland) The water activity was measured at 25°C and the equilibrium state of humidity over the sample was reached during 30 minutes.

RESULTS AND DISCUSSION

Figure 1 shows the time dependence of the temperature of rice in the homogenising steriliser. The temperature was measured on the heat exchange surface and the shaft of the apparatus. The differences between the temperatures on the wall and at the shaft during the holding period were 4°C at the most and steadily decreased during the holding time. The temperature sensor at the shaft is not in contact with the rice when the batch is 10 kg. The figure shows instances of sampling during the holding period that are manifested by a slight decrease of temperature.

Table 1 gives the values of the moisture content at the start and the end of decontamination. It can be seen that during the treatment the moisture content decreased considerably.

Table 1. Changes in the moisture content and water activity of rice during treatment

Sample	Moisture content (% wet basis)	Water activity (–)
N_1 – before treatment	13.83	0.725
N_5 – at the end of sterilisation	2.66	0.033

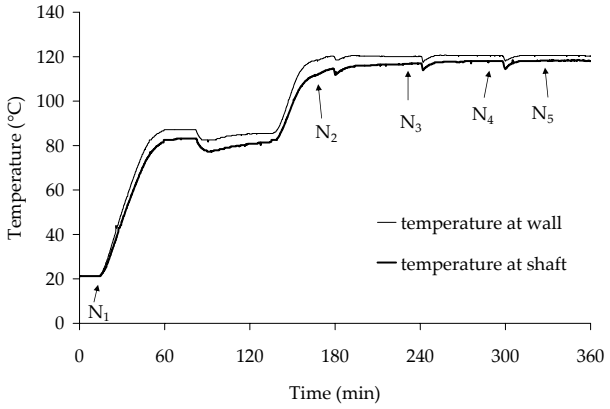

Figure 1. Time–temperature dependence during rice sterilisation at 120°C

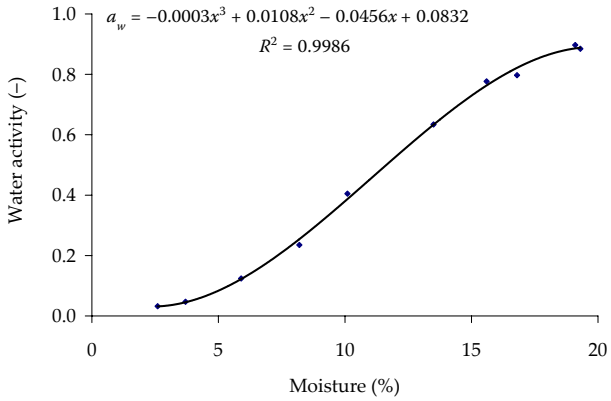

Figure 2. Desorption isotherm of the rice

Table 2. Quantitative presence of vegetative cells and spores of *Bacillus cereus* CCM 2010 as a function of holding time at 120°C

Sample label – description	Number of vegetative cells[1]	Number of spores[2]
N_0 – inoculum	6.00×10^5/ml	4.99×10^4/ml
N_1 – rice, starting state after drying at 30°C (before commencing heating)	5.79×10^2/g	4.27×10^2/g
N_2 – rice at the start of the holding period	3.64×10^2/g	2.86×10^2/g
N_3 – rice after 1 h of holding at 120°C	negative*	negative*
N_4 – rice after 2 h of holding at 120°C	negative*	negative*
N_5 - rice after 3 h of holding at 120°C	negative*	negative*

[1]calculated from the number of confirmed colonies grown on the medium MYP; [2]calculated from the number of confirmed colonies grown on the medium PCA; *below the limit of determination of the standard method used, $\leq 10^2$ CFU/g

Desorption isotherm of rice was predicted (Figure 2). This graph was used for the prediction of water activity of the rice samples during the treatment (Table 1). It is apparent from Table 1 that water activity also decreased considerably during the decontamination procedure.

Table 2 shows the results of microbiological analyses of the samples of inocula and contaminated rice during decontamination.

The results show that the inoculum contained significant numbers of both vegetative cells and spores of *B. cereus* which suited the purpose of the experiment. Losses occurred during contamination because theoretically the numbers in contaminated rice should have been only ten times smaller (1 kg of inoculum to 9 kg of rice). These losses may be due to grinding of the surface of rice (where the greatest contamination is expected) during homogenisation and drying at 30°C. Equally, the soaking ability of rice could have an influence. The starting concentrations obtained are of the same order as those occurring in practice and in other products.

An important finding is that already after one hour of holding the temperature at 120°C neither vegetative cells nor spores of *B. cereus* were found. This means that during one hour of holding the temperature at 120°C there was at least two decimal reduction in the numbers of vegetative cells and spores.

CONCLUSIONS

Decontamination of rice from vegetative cells and spores of *B. cereus* occurring in the order of 10^2 can be achieved by heating to 120°C and holding this temperature for one hour. It is likely that it would be possible to remove even higher concentrations of micro-organisms if it were possible to reach more massive starting contamination. The homogenising steriliser, which mixes heated

layers of powdery foods intensively, is well suited for the decontamination.

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