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STANISŁAW ZALESKI
KRYSTYNA SOBOLEWSKA-CERONIK
EDWARD CERONIK
ELŻBIETA DACZKOWSKA
ELŻBIETA MAZUR
TERESA BOGUSŁAW
WŁODZIMIERZ ŻEREK

EFFECTS OF BALTIC FISHES FRESHNESS ON THERMAL RESISTANCE OF BACTERIAL SPORES

I. THERMAL REDUCTION OF *BACILLUS SUBTILIS* SPORES SUSPENDED IN HEAT DENATURATED EXTRACTS FROM MEAT OF HERRING AND COD OF DIFFERENT FRESHNESS

Institute of Marine Food Technology, Agricultural University, Szczecin

Key words: *Bacillus subtilis*, fish meat, thermal resistance of spores.

The aim of the study was to determine the effect of freshness of two Baltic industrial species, herring and cod, on the course of thermal inactivation of bacterial spores. The study was based on a premise that the deteriorating freshness is represented by heat denaturated extracts of fish meat from specified periods of icing at 0°-4°C for 12 days. The extracts were used as media suspending spores of *Bacillus subtilis* strain No. 32 (Warsaw) during heating at 95°C. The survival curves were plotted with the test-tube technique with the use of nutritive agar for recovery of the spores.

The investigations proved that: 1) thermoresistance of *Bacillus subtilis* spores in herring and cod meat extracts increases gradually with the extension of storage on ice and reaches maximum on the 12th day of storing; 2) the gradual increase in thermal resistance reflected by the extended heating time required to obtain 99.99% reduction of the initial population of spores results from delay in exponential reduction of spores effected by the changing degree of thermal activisation of spores and the growth of the number of spores surviving identical heating periods as a function of the ongoing spoilage; 3) the observed increase of thermal resistance of spores can be considered as a development of protective action that could be due to accumulation in the spoiling fish meat of certain thermoconstant component or components protecting the spores in the course of heating.

INTRODUCTION

Recently, the extent of heat treatment applied in thermal preservation of foods is generally based on the thermal resistance of spores of pathogenic and spoilage micro-organisms and their spores exhibited in optimal conditions e.g., by using in heating tests the suspending and recovery media yielding the highest number of survivors. This way of reasoning introduces high safety factor to processing parameters which is especialy important for public health [17]. On the other hand, trend toward application of milder heat treatment in processing of foods is observed. It is proved by intensive studies on possibilities of germination and outgrowth of heat injured bacterial spores in relevant food substrates. In this sense, food products are individually considered as containing natural and introduced inhibitors and/or stimulating compounds, and determined level of water activity [4, 10, 13, 18].

Relatively early in thermobacteriological studies of spores, influence of such factors as chemical composition of sporulation, suspending and recovery media on thermal resistance was recognized; and the literature contains abundancy of research works devoted to determination of influence of different inorganic and organic compounds on thermal resistance of bacteria, and large part of them is dea ling with foods [8, 12, 14, 19].

Fish processing industries use as well mild heat treatment (pasteurization range) e.g. precooked fish production or smoking, as more severe heating during sterilization of canned fish. In Polish fish industry raw material is of Baltic Sea origin and enters processing as an iced fish, or it is supplied by deep sea fishing in a frozen state.

During spoilage of the iced fish, number of putrefactive bacteria is increasing and as a result of their activity, accumulation of degradation products in fish muscles takes place [6, 15]. Action of these compounds on heated spores in not exactly defined yet. This study was undertaken to determine, how the degree of freshness of the two species of Baltic fishes influences order of thermal inactivation of bacterial spores, with the assumption, that decreasing freshness is represented by heat denaturated extracts prepared out of fish meat taken in defined intervals from experimental stock stored in ice at 0-4°C up to 12 days, and consequently, these extracts will serve as suspending media in heating tests.

MATERIALS AND METHODS

1. PREPARATION OF STOCK SPORE SUSPENSION

Bacillus subtilis strain No. 32 (PZH Warsaw) was selected as a test organism. Nutrient agar 2.5% was used throughout the experiments as culture medium (slants), sporulation and the recovery medium (plates).

Spore crop was hervested after 8 days incubation at 30°C and suspended in distilled water. Crude suspension was cleaned by repeated centrifugation and washing with destilled water — three times at 1.000 x g. Final suspension was stored at 2°C. Viable counts of this suspension were made after heat shock 15 min. at 70°C. Concentration of stock spore suspension was 7×10^6 spores/ml.

2. PREPARATION OF MEAT EXTRACTS FROM BALTIC COD AND HERRING

Freshly caught fishes were washed, iced and stored at the temperature range 0-4°C. Duration of storage did not exceed 12 days. At the desired interval of storage time, samples of fish were withdrawn to prepare meat extracts. Fishes were filleted and skinned out, meat was next minced, placed in equal portions at 2000 ml Erlenmeyer flasks, covered with 20 mm layer of soya oil and next subjected to autoclaving at 121°C for 45 min. After autoclaving, water layer was filtered out, fatty residues were discared afer the overnight refrigerating in a separator, and obtained meat extracts were finally distributed to flasks in 100 ml portions, and sterilized 10 min. at 121°C. Prepared extracts were stored under refrigeration.

In case of herring, there were two versions of extracts — with and without addition of sodium chloride. Salted extracts of herring were prepared under indentical conditions as the others with this exception though, that before autoclaving, gutted fishes were salted by standard dipping in 20% NaCl solution for 4 min., under constant proportion brine/fish 2.5; 1 (v/w). As a result of changing degree of freshness of fish the obtained meat extracts contained diferent amounts of salt.

3. TESTING OF THERMAL REDUCTION OF *BACILLUS SUBTILIS* SPORES SUSPENDED IN FISH MEAT EXTRACTS

Appropriate portions of stock suspension of spores were introduced to meat extracts, to get final concentration of 5.000 spores per ml of herring extracts and 10.000 spores per ml of cod extracts. Prepared test suspension were next distributed in 1 ml amounts to test tubes 8×100 mm and tubes were hermetically stoppered. Totally immersed tubes were heated at selected time intervals, in thermostatically controlled water bath at 95°C. During heating tubes were closed in brass statives [20]. Times of heating were corrected for temperature lag by addition of 1.5 min to each. Three tubes were heated in each time-temperature combination. After 10 min. cooling in iced water, content of tubes was plated onto the surface of 2.5% nutrient agar plates and incubated 10 days at 37°C. After that, colonies formed by surviving spores were counted.

RESULTS

Percentage of survival of heated spores was computed from colony counts and was based on the initial number of spores determined after 15 min heath shock of suspension at 70°C. Each presented precentage of survival was determined from triplicate sample. Obtained percentage values were next plotted against appropriate times of heating on semilog paper, and survival curves were drawn to be best fit experimental points — Fig. 1, 2 and 3.

As can be seen, all those curves shows nonexponential order of inactivation at the beginning of heating, for their initial parts are curved. For this reason it was decided, after Roberts and Hitchins [12], that thermal reduction time required to reduce the initial population of spores up to 99,9% would be the best value expressing differences in thermal in-

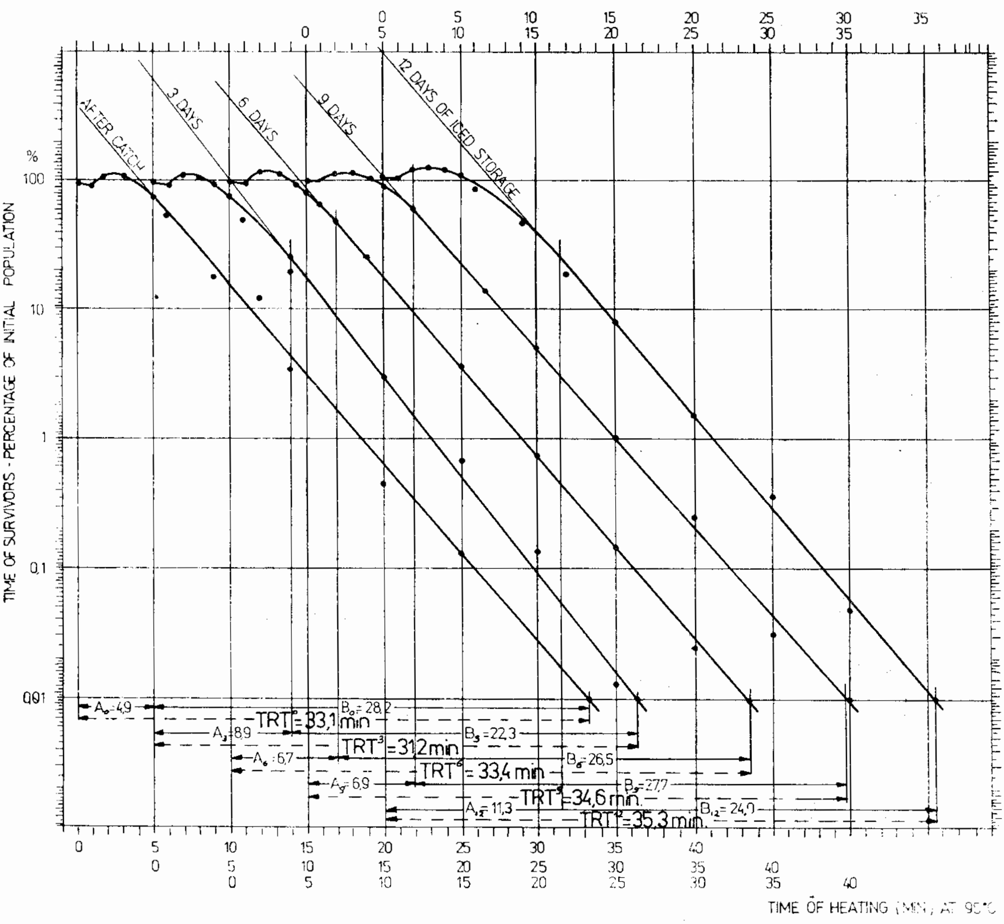

Fig. 1. Survivor curves of *Bacillus subtilis* spores suspended in meat extracts from Baltic herring of different freshness. Initial population 3×5.000 spores per ml.

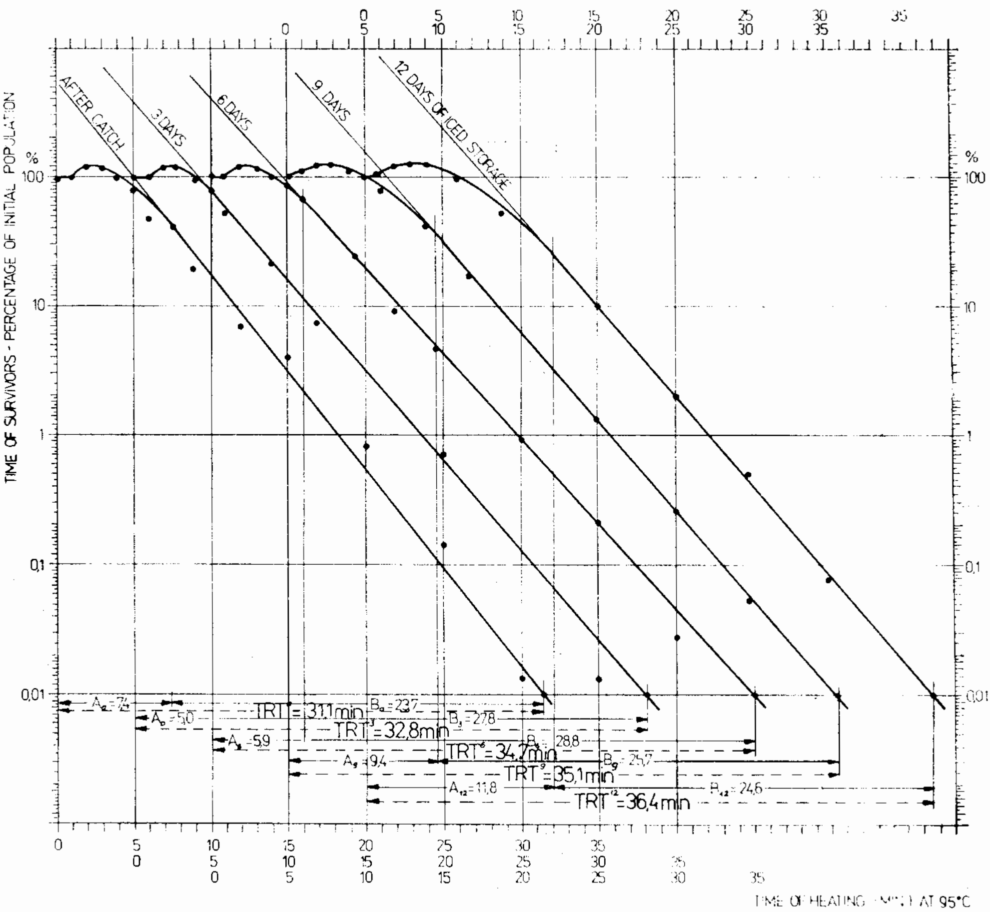

Fig. 2. Survivor curves of *Bacillus subtilis* spores suspended in meat extracts from herring of different freshness, with addition of NaCl. Initial population 3×5.000 spores per ml.

activation of spores in different extracts. It was also decided to express differences of inactivation order by use of additional factor e.g., proportion between heating time A (min) corresponding to curved portion of survivor curve, and TRT 99.99% (min) value. Obtained values of TRT-s and corresponding A/TRT proportions for extracts of herring and cod are shown in Table 1, 2 and 3.

Bacillus subtilis spores suspended in herring extracts prepared out of the fish stored 0-12 days, showed different resistance to heat dependently on the spoilage stage. Appropriate survivor curves are shown on Fig. 1, and comparison of TRT-s contains Table 1.

It is shown, that the largest TRT value corresponds with the 12th day of storage; on the other hand, the least value of TRT is observed 3rd

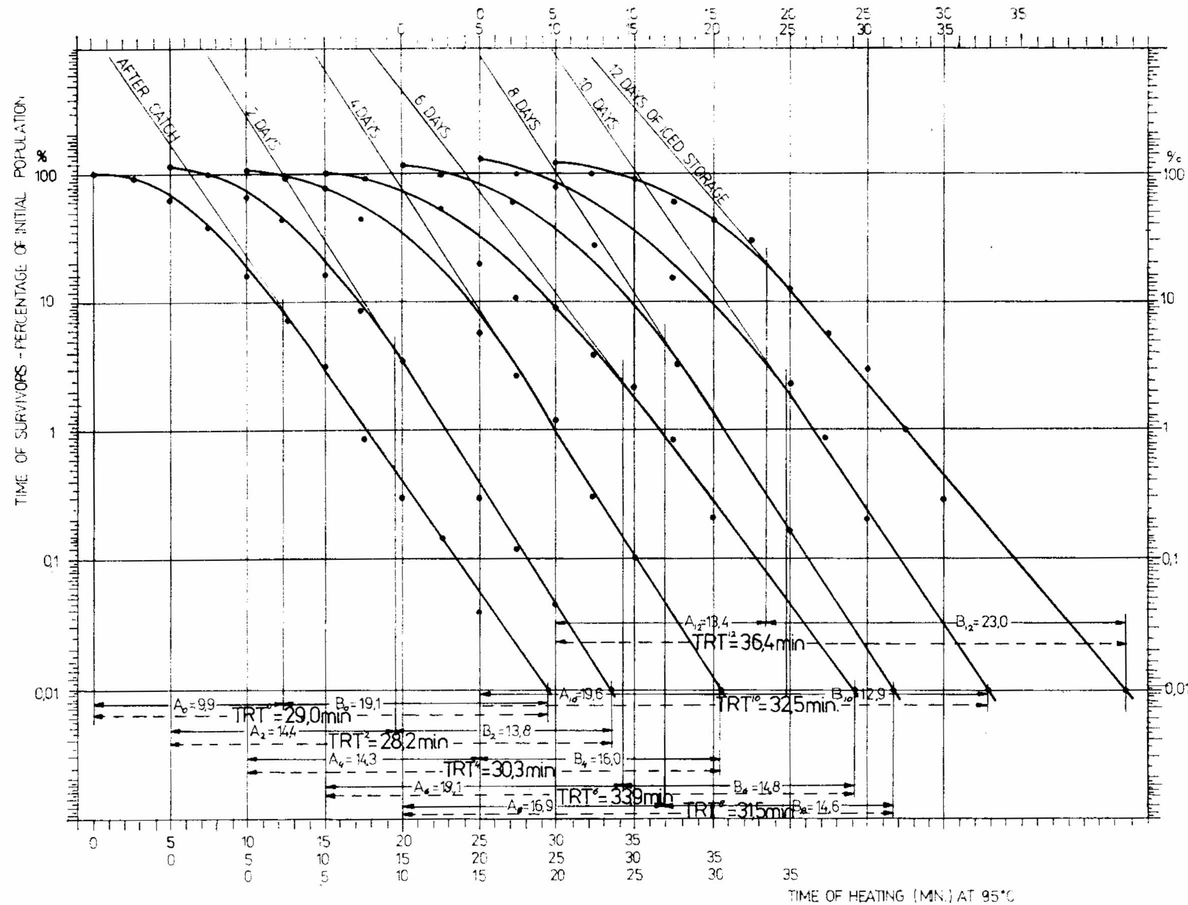

Fig. 3. Survivor curves of *Bacillus subtilis* spores suspended in meat extracts from Baltic cod of different freshness. Initial population 3×10.000 spores per ml.

T a b l e 1. Heat resistance of *Bacillus subtilis* strain No. 32 (PZH, Warsaw) spores suspended in extracts from Baltic herring of different freshness. Spores concentration 5.000 per ml. Temperature of heating 95°C

Storage time (days)	pH of prepared extracts	Description of semilog survivor curve			
		delay of exponential reduction — curved portion A (min.)	exponential portion B (min.)	thermal reduction time 99.99% A+B (min.)	A/TRT proportion
0	6.75	4.9	28.2	33.1	0.15
3	6.80	8.9	22.3	31.2	0.29
6	6.90	6.9	26.5	33.4	0.21
9	6.90	6.9	27.7	34.6	0.20
12	7.10	11.3	24.0	35.3	0.32

T a b l e 2. Heat resistance of *Bacillus subtilis* strain No. 32 (PZH, Warsaw) spores suspended in salted extracts from Baltic herring of different freshness. Spores concentration —5.000 per ml. Temperature of heating 95°C

Storage time (days)	pH of prepared extract	NaCl content of prepared extracts m/v (%)	Description of semilog servivor curve			
			curved portion (delay of exponential reduction A (min)	exponential portion B (min)	thermal reduction time 99.99% A+B (min)	A/TRT proportion
0	6.60	1.57	7.4	23.7	31.1	0.24
3	6.65	1.86	5.0	27.8	32.8	0.15
6	6.80	2.40	5.9	28.8	34,7	0.17
9	6.85	2.85	9.4	25.7	35.1	0.27
12	6.90	3.23	11.8	24.6	36.4	0.32

T a b l e 3. Heat resistance of *Bacillus subtilis* strain No. 32 (PZH, Warsaw) spores suspended in extracts from Baltic cod of different freshness. Spores concentration — 10.000 per ml. Temperature of heating 95°C

Storage time (days)	pH of prepared extracts	Total vola ile bases content of meat mg % N	Description of semilog survivor curve			
			curved portion (delay of exponential reduction) A (min)	exponential portion B (min)	TRT for 99.99% destruction A+B (min)	A/TRT proportion
0	6.10	4.90	9.9	19.1	29.0	0.34
2	6.20	5.32	14.4	13.8	28.2	0.51
4	6.25	5.60	14.3	16.0	30.3	0.47
6	6.30	9.20	19.1	14.8	33.9	0.56
8	6.30	17.10	16.9	14.6	31.5	0.54
10	6.70	24.10	19.6	12.9	32.5	0.60
12	6.75	23,52	13.4	23.0	36.4	0.37

day of storage. With small exception (the 3rd day of storage) it could be stated, that while freshness of herring decreases, the corresponding values of TRT-s 99.99% are continously increasind. A/TRT proportion shows variations in different intervals of storage, and the largest lag of exponential reduction of spores corresponds with maximal obtained TRT value. An additional demonstration of inactivation order dependence on storage time of herring is given on Fig 4A and Fig. 5A, which shows continuous increase of percentage of heat activated and surviving spores after identical heat treatment, as a function of storage time. Obtained curves show good correlations with increased values of TRT 99.99 W.

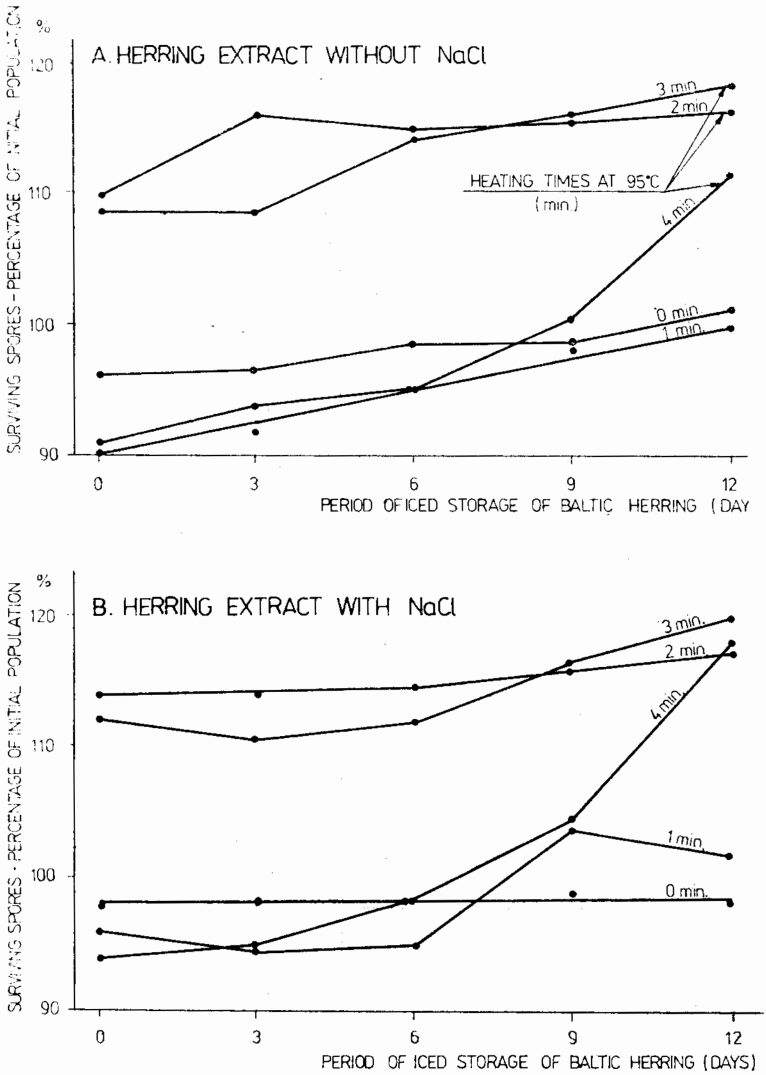

Fig. 4. Heat activation of *Bacillus subtilis* spores as a function of storage time; A. Herring extracts without NaCl. B. Herring extract with NaCl.

Similar tendencies are observed for spores suspended in salted herring extracts, containing from 1.57 to 3.23% of of NaCl — Fig. 2, Table 2, Fig. 4B and Fig. 5B. In this case, the largest TRT 99.99% value corresponds with the longest period of herring storage and besides, while fresh-

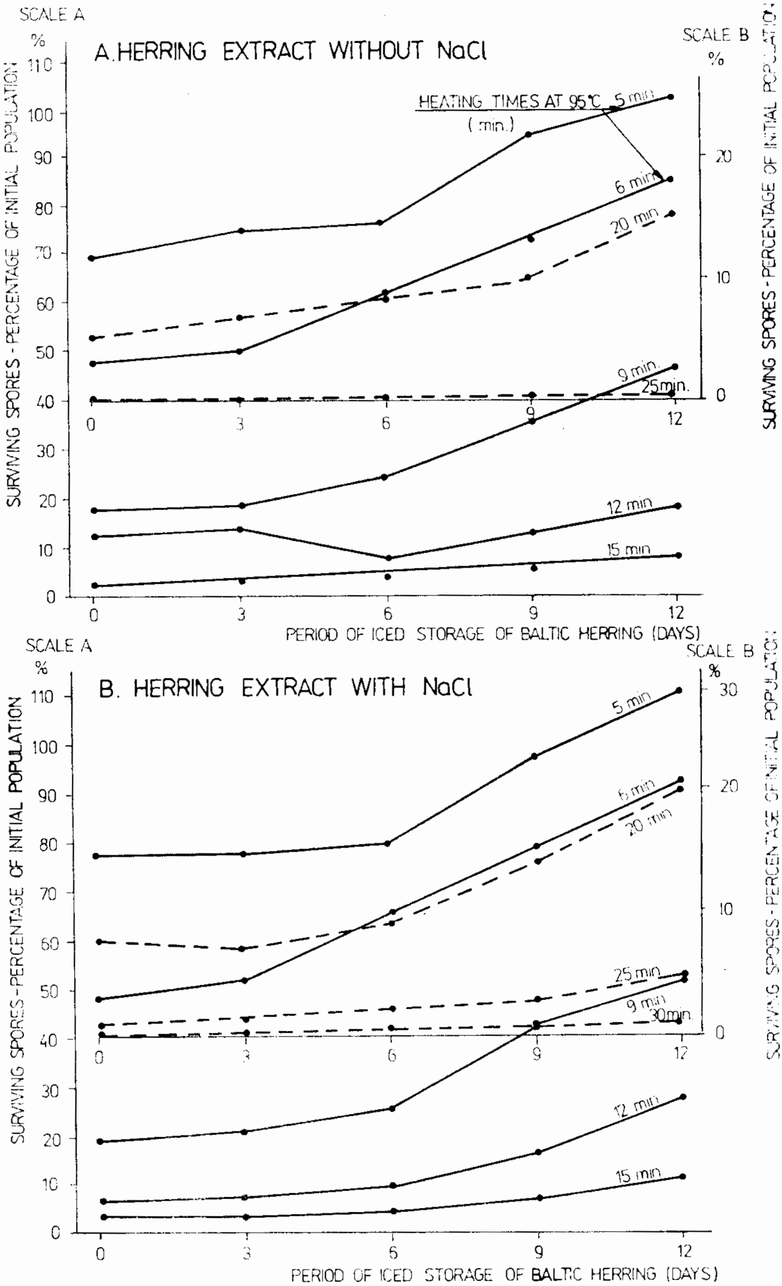

Fig. 5. Surviving of *Bacillus subtilis* spores as a function of storage time; A. Herring extract without NaCl. B. Herring extract with NaCl.

ness decreases, TRT 99.99% values are continously increasing. This phenomenon is a result of increasing degree of spores activation (Fig. 4B) as well as of the percentage survival increase after identical heating parameters (Fig. 5B) in function of storage time. It should be stressed, that inactivation order in unsalted and salted herring extracts show close resemblance, which could be easily seen by comparison of TRT-s values, increase of percentage survival as a function of storage time and corresponding values of A/TRT 99.99% proportion.

Thermal reduction of *Bacillus subtilis* spores suspended in the cod extracts, generally, shows similar correlation, as found for the herring extracts. The largest TRT 99.99% value — 36.4 min. is also observed after 12 days of storage. On the other hand additional peak of resistance is observed after 6 days of storage, with TRT 99.99% amounting 33.2 min. Generally, in extracts prepared between 0 and the 6th day of storage, spores show continously increasing resistance, slight decrease of its in extracts at the 8th day, and after that, at the 10th and the 12th day increase of resistance of maximal value (Fig. 3 and Table 3).

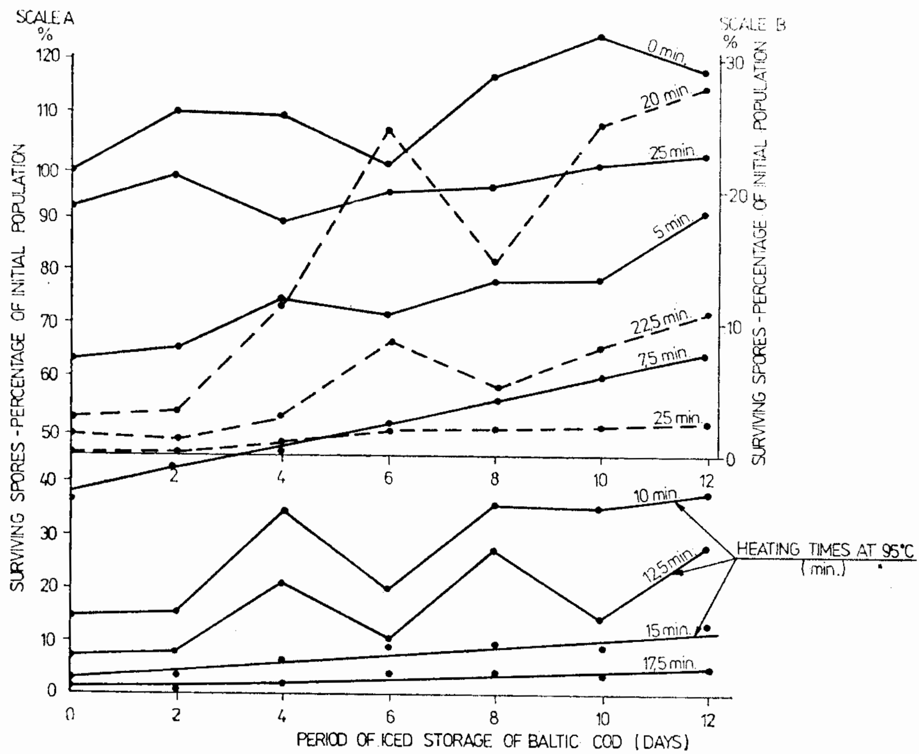

Fig. 6. Surviving of *Bacillus subtilis* spores suspended in cod extracts as a function of storage time.

In cod extracts, lag of the exponential reduction of spores (A/TRT 99.99% values — Table 3) is more expresively marked, than in herring extracts. In cod extracts, there is also slightly different picture of activation degree and increase of the percentage survival after identical times of heating, as a function of decreasing quality (Fig. 6).

DISCUSSION

In this study, only a fish freshness was changing factor, and observed variations of inactivation order and degree of spores activation should be treated as the reflection of complex chemical changes accompanying fish spoilage [15].

An addition of sodium chloride to the herring extracts, which was made in one set of experiments by the same method as used habitually in fish canning. Regardless of some resulting differences in content, NaCl has shown rather minor effects on observed thermal resistance of spores.

Addition of NaCl had an insignificant effect on thermal resistance of the investigated spores or no effect at all. This is analogues with the results reported by others [5, 11], and particularly with determinations of *Bacillus stearothermophilus* SS 1518 in homogenates of mackerel and herring muscle tissue [16] Here, an increase of NaCl from 0 to 3% in herring homogenates showed only a minor effect on the course of thermal inactivation. In the mackerel homogenates, however, the protective action of salt was clearly marked. Taking into account the results obtained in the older and the present studies, it is possible to maintain that the effect of NaCl on bacterial spores during heating depends largely on the nature and properties of the suspension medium [3, 7, 9, 19].

Prepared fish extracts have shown some variations of pH value and this variability was dependent on the fish freshness. In case of the herring extracts, pH has changed from 6.6 to 7.1 and in cod extracts from 6.1 to 6.8. It might be supposed, that those variations have not posessed any definite influence on the observed resistance of spores, because their values were always in the range optimal for thermal resistance of spores [4].

It was shown [2] that undenaturated protein compounds and living vegetative cells of Gram positive bacteria present in suspending medium, exert protective effects on heated spores. An opinion was presented, that this effect is due to a partial adsorbtion of an activation energy in suspending medium by undenaturated protein molecules, which could be proved by lack of protection by heat denaturated proteins, living cells of Gram negative bacteria, and dead cells of Gram positive bacteria.

It was shown that differences in thermal resistance of *Cl. botulinum*

Type E spores in fish substrates were rather dependent on physical state of suspending medium, because its thermal resistance was significantly higher in raw fish mince, than in precooked fish mince [1]. Because this effect was duplicated by the use of egg-white, which coagulates rapidly at the same temperatures as tested spores, authors concluded, that observed differences have not resulted from a heat labile factor in raw fish, which stimulates outgrowth of heat injured spores.

In our study, heat denaturated extracts, made out of fish of different freshness showed relative, protective action on suspended spores of *Bacillus subtilis*. It means, that when iced storage proceeds up to 12 days, decrease of freshness makes longer heating necessary to obtaine assumed level of 99.99% reduction of the initial population, suspended in extracts, representing defined period of storage. On the other hand, in extracts representing fish shortly after catch, shorter heating was necessary to obtain the same level of reduction.

Obtained results may indicate, that when the spoilage of herring and cod muscles proceeds, accumulation of compound or compounds takes place, content of which is a function of storage time. Their presence in prepared extracts results in protective action of suspended spores during heating, despite of earlier heat treatment applied during preparation of extracts. It might be supposed, that similar action of these compounds could be observed during industrial processing of herring and cod.

CONCLUSIONS

1. Thermal resistance of *Bacillus subtilis* spores suspended in muscle extracts of Baltic herring and cod gradually increases with iced storage period and reaches its maximal value at the end of storage, on the 12th day.

2. Gradual increase of thermal resistance of spores demonstrated by extending of Thermal Reduction Time required for 99.99% destruction of initial spores population, is a result of delays of exponential reduction caused by changing degree of heat activation of spores, and increase of survivors number after identical heating, as a function of developing spoilage.

3. Observed increase of spores resistance might be considered as a development of relatively protective action, which could result out of the accumulation in spoiling muscles of some heat resistant stable compound (a), which protects spores during heating.

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Authors address: Kazimierza Królewicza 3, 71-550 Szczecin.

S. Zaleski, K. Sobolewska-Ceronik, E. Ceronik, E. Daczkowska, E. Mazur, T. Bogusław, W. Żerek

WPŁYW ŚWIEŻOŚCI RYB BAŁTYCKICH NA CIEPŁOOPORNOŚĆ SPOR BAKTERYJNYCH

I. REDUKCJA TERMICZNA SPOR *BACILLUS SUBTILIS* ZAWIESZONYCH W ZDENATUROWANYCH CIEPLNIE WYCIĄGACH Z MIĘSA ŚLEDZIA I DORSZA O RÓŻNEJ ŚWIEŻOŚCI

Instytut Technologii Żywności Pochodzenia Morskiego, AR, Szczecin

Streszczenie

Celem badań było określenie wpływu świeżości dwóch przemysłowych gatunków ryb bałtyckich — dorsza i śledzia na przebieg inaktywacji termicznej spor bakteryjnych. Założono przy tym, że pogarszająca się świeżość będzie reprezentowana przez zdenaturowane cieplnie wyciągi przygotowane z mięsa ryb z określonych czasokresów przechowywania w lodzie, w temperaturze 0-4°C, trwającego do 12 dni. Wyciągów tych użyto jako środowisk zawieszających spory *Bacillus subtilis* szczepu nr 32 (PZH, Warszawa) podczas ogrzewania w 95°C. Krzywe przeżycia spor wyznaczono techniką próbówkową, stosując do odzyskania spor agar odżywczy.

Badania wykazały, że: 1) ciepłooporność spor *Bacillus subtilis* zawieszonych w wyciągach z mięsa śledzia i dorsza stopniowo wzrasta wraz z okresem przechowywania w lodzie i osiąga swą maksymalną wartość przy końcu składowania w 12 dniu; 2) stopniowy wzrost ciepłooporności przejawiający się wydłużaniem czasu ogrzewania potrzebnego do uzyskania 99,99% redukcji inicjalnej populacji spor jest wynikiem opóźnienia redukcji wykładniczej spor spowodowanego przez zmieniający się stopień cieplnej aktywizacji spor i wzrost ilości spor przeżywających identyczne czasy ogrzewania w funkcji postępującego zepsucia; 3) obserwowany wzrost oporności termicznej spor może być rozpatrywany jako wystąpienie względnego oddziaływania ochronnego, które może być wynikiem nagromadzania się w psującym się mięsie ryb pewnego ciepłostałego składnika lub składników, które chronią spory podczas ogrzewania.