Talaromyces flavus ascospores as bioindicators

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INTRODUCTION

Most of heated food is overheated because there is no way to know exactly which treatment the product had practically received. The theoretical calculations keep a safety margin on the treatment. According to the chemical and physical properties of the treated food, for the same time temperature treatment, the effect will be different. That is the reason why a heat-process has to be over the theoretical minimum to be safe, so food is mostly over-heated. That phenomenon could be avoid if a way of evaluation of the real treatment the food had submitted, could be found out. So, the food value and the sensorial quality could be optimized.

As it is already used for sterilization process, alginate beads with whole cells (bacteria or bacteria spores) immobilized could be introduced into the food during the heat-processing. Then, the real heating can be calculated by counting the heat-destruction of bacteria into the beads. The problem is to find out a good microbiological indicator for the pasteurization range. Bacteria spores used for the sterilization-processes are too heat-resistant to be destroyed in the range of temperature 80°C to 90°C. So, we would like to try to work with mould ascospores which are much less heat-resistant than bacteria spores.

Many papers on ascospores heat-resistance outline the non-logarithmic curve of heat-destruction. This phenomenon makes inappropriate the use of Bigelow's formulas for logarithmic decreases. In 1970, ALDERTON G. and N. SNELL (2) finalized an experimental modified formula for non-logarithmic curves of heat-destruction. Since their experiments, several studies about mould ascospores heat-resistance had used this modified formula to interpret the curves and to calculate some values which could be compared to each other.

In 1979, Henri G. BAYNE and H. David MICHERNER (3) compare the experimental formula for non-logarithmic curves with the formula for logarithmic curves. They have used five strains of *Byssochlamys fulva*. One Log of destruction is representative for an non-logarithmic curve because it is not straight. At least three Logs must be counted. The 3D value (time to kill 99,9% of the initial concentration at a given temperature) is compared to the 3Log value which has the same meaning than the 3D value but is used only for non-logarithmic curves. The results are:

<table>
<thead>
<tr>
<th>pH</th>
<th>3Log experimental</th>
<th>3Log observed</th>
<th>3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH3.6</td>
<td>2 to 41 min</td>
<td>4 to 45 min</td>
<td>4 to 36 min</td>
</tr>
<tr>
<td>pH5.0</td>
<td>1.2 to 51 min</td>
<td>4 to 51 min</td>
<td>4 to 34 min</td>
</tr>
</tbody>
</table>

Table 1.
The results outline that 3Log calculated and 3Log observed are almost equal (1 or 2 min of difference), and that 3D is always lower that 3Log. Nevertheless, 3D is used as the pasteurization value in heat-processing. That means the heat-treatment used usually is not enough to destroy efficiently mould ascospores. Thus, those *Byssochlamys* ascospores are responsible for numerous problems in canned fruits or jams. The same kind of problems are found with *Talaromyces flavus*.

Therefore, it is very important for us to use the closest formula to reality. We used the experimental formula for non-logarithmic curves of ALDERTON and SNELL (1970).

The formula is \((\text{Log No} - \text{Log N})^* = k^*t + C\) for non-logarithmic curves instead of \((\text{Log No} - \text{Log N}) = k^*t + C\) for logarithmic curves. The factor "a" is used to linearize the non-logarithmic curve.

Several studies had been done on *Byssochlamys nivea* or *fulva* but few others have been done on *Talaromyces flavus*. The results are:

<table>
<thead>
<tr>
<th>From</th>
<th>Temperature</th>
<th>(1/k) or D*</th>
<th>z</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEUCHAT, 1986 (4) on 2 strains</td>
<td>91°C 88°C</td>
<td>2.9 to 11.7 min 3.5 to 22.3 min</td>
<td>5.2°C to 12.9°C</td>
</tr>
<tr>
<td>SCOTT and BERNARD, 1987 (11)</td>
<td>90.6°C 88°C</td>
<td>2.2 min 7.8 min</td>
<td>5.2°C</td>
</tr>
<tr>
<td>KING and WHITEHAND, 1990 (10) on strain NRRL 13641</td>
<td>90°C 80°C</td>
<td>6 min (a=0.181) 191 min (a=0.261)</td>
<td>6.7°C</td>
</tr>
</tbody>
</table>

* D is calculated on the straight part of the curve, D value for the two first datas.

Table 2.

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Heating temp (°C)</th>
<th>a*</th>
<th>k*</th>
<th>C</th>
<th>1/k</th>
<th>3 log</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL 13535</td>
<td>80</td>
<td>0.4053</td>
<td>0.01549</td>
<td>-0.05552</td>
<td>63</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>0.2020</td>
<td>0.03977</td>
<td>-0.04023</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
<td>NRRL 13535</td>
<td>90</td>
<td>0.2534</td>
<td>0.1511</td>
<td>-0.05232</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>NRRL 13536</td>
<td>80</td>
<td>0.3233</td>
<td>0.007736</td>
<td>0.1819</td>
<td>113</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>0.4188</td>
<td>0.45842</td>
<td>-0.04238</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.2534</td>
<td>0.2496</td>
<td>0.06275</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>NRRL 13537</td>
<td>80</td>
<td>0.7258</td>
<td>0.01049</td>
<td>-0.06314</td>
<td>96</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>0.3060</td>
<td>0.03985</td>
<td>-0.02408</td>
<td>26</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.5499</td>
<td>0.3145</td>
<td>-0.06918</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>NRRL 13538</td>
<td>90</td>
<td>0.4150</td>
<td>0.3010</td>
<td>-0.06149</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

* a*—The reciprocal slope of the plot of log (log No - log N) against log time. This is a rearrangement of the basic formula \(\text{log No} - \text{log N})^* = k^*t + C.\)

* k*—Values were determined from the slope of the linearized plot using the previously determined a value in the basic formula and \(C\) is the intercept.

* Time for decrease in 1 k value by 1 log(s), and calculated time for 3 log(s) drop in viable count.

Table 3 from KING and HALBROOK (1987) (9)
This project has three aims:
- To improve the work already done in ascospores harvesting with a view to obtain the purest suspension as possible;
- To achieve some pasteurization values by heat-destruction of free ascospores;
- To compare those pasteurization values with the values found on immobilized ascospores.

This project is a part of an European project to improve safety and quality of heat preserved products. The purpose is to find out different materials to be a TTI (Time Temperature Indicator). This TTI must be easy and fast to prepare, it must quantify the impact of the process, it must be easy to read and to use.

Figure 1.
MATERIALS AND METHODS

I. Presentation of our strain

The moulds which produce ascospores are classified within the class *Ascomycetes*. Inside this class are fungi like yeasts and hyphae-forming moulds which all produce ascospores. Those are produced generally in groups of 8, very seldom in groups of 16 or 32. The eight ascospores are produced enveloped in "closed bag" called ascus (pl: asci). Most of moulds, (no yeast and no *Byssochlamys*) can produce asci enclosed within larger bodies. Those bodies hold a large number of asci and their diameter can reach 40 or 80 μm. They are called fruit bodies. Yeast ascospores are much less heat-resistant than mould ascospores which are responsible for many damages of canned fruits, heat-preserved fruit juices and jams (P. ADAMEK and U. RÖNNER, 1993) (1). Ascospores heat-resistance is influenced by composition of growth medium and heating medium, by the age of the culture, by the growth conditions (M.L.A. CASELLA and al.,1990) (6) and (KING and WHITEHAND, 1990) (10).

Figure 2.
**Genus Talaromyces:**

The anamorphic stages constitute genus *Penicillium*. *Talaromyces* is known for the production of yellow or white fruits bodies with fine structure of small hyphae on the outside. The most common specie is *Talaromyces flavus* (ADAMEK and RÖNNER, 1993) (1). The specie *Talaromyces flavus* has been divided in two subspecies by STOLK and SAMSON in 1972: *Talaromyces flavus var. flavus* which ascospores are ellipsoidal and 3,5-5,0 * 2,5-3,2 μm and *Talaromyces flavus var. macrosorus* which ascospores are also ellipsoidal and bigger: 5-6,5 * 2-2,5 μm. Conidia-spores seldom produced, can form greyish green sectors. They are 2,2-3,5 * 2,0-2,5 μm (K.H. DOMSH and al.,1980) (7).

The best growth of *T. flavus* is at 37°C and it doesn’t grow anymore at 5°C. A degradation of starch, cellulose and laminarin can be observed by action of 1,3-β-D-glucanase. Some proteinas can be formed, hydrocarbon from fuel oil utilized. This fungus also shows an antibiotic activity against bacteria and *Streptomyces* and produce vermiculine which is an antibiotic with antibacterial and antiprototyzoal properties. It can inhibit the growth of few others moulds. (K.H. DOMSH and al., 1980) (7).

*T. flavus* can be found in soil from many countries and in fruits. The strain we used has been extracted from fruit. It is a *Talaromyces flavus var. macrosorus*, CBS 317.63, or IMI 197478 AC. STOLK.

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Fig. 334. *Talaromyces flavus*. a. Penicilli and conidia; b. development of ascogonia and antheridia; c. chains of asc; orig. A. C. Stolk (from 5593); d. mature asc and ascospores; a. and d. orig. J. Veerkamp.

Figure 3 from DOMSH and al., 1980 (7)
II. Harvesting

The culture was harvested after 4 weeks growing on OMA (oat meal agar) at the room temperature (20°-22°C). The inoculation was made from a culture growing on MEA (malt extract agar with 2% of glucose) at the same temperature which is inoculated from another culture growing on OMA.

The stages of harvesting were always the same. The method had been used in most of papers. Therefore, some stages could be added to perfect the method with the view to obtain the ascospores suspension the purest as possible. We have changed some stages conditions according to the results we obtained. So, the main stages are:

- harvesting with 4 to 10 ml of sodium chloride (0.85%) from the petridish;
- soft homogenization with the magnetic stir in sodium chloride during 45 min;
- strong homogenization in the mixer Sorvall (omni mixer) to break most of hypha;
- filtration through sterile glass whool filters to take off the biggest pieces of hypha;
- successive centrifugations in sodium chloride to clean the suspension (3-4 centrifugations in a Sorvall RC-5B Refrigerated Superspeed Centrifuge by Du Pont Instruments);
- low freezing of the suspension to put it in the French Pressure Cell;
- the suspension in the freezed French Pressure Cell and the whole equipment in the freezer -40° or -63°C for the night;
- the suspension goes through the French Pressure Cell under high pressure to break down the asci and release the free ascospores;
- centrifugation of the suspension to concentrate it to 10^5 or 10^6 ascospores/ml.

III. Immobilization of ascospores: method borrowed from C. BUCKE, 1987 (5)

We used a middle-density alginate (Manucol-DM, from KELCO company, No E.5774) at the concentration of 5%. The successive stages were:

- 1 ml of the ascospores suspension the most concentrated as possible were mixed with 9 ml of alginate 5% because 3% was not enough after sterilization which changed it to a lower viscosity;
- about 160 alginate beads of a 0.0625 ml were made with a small syringe of 1 ml by dropping them in a bath of calcium chloride 4%;
- in each tube, 8 alginate beads, which is 0.5 ml, were heated during different time intervals in sodium chloride 0.85% (the same heating medium than the free ascospores);
- after the heat-treatment, these 9 alginate beads (0.5 ml) were solubilized in 4.5 ml of sodium citrate 5% by stirring with the magnetic stir during 25 min;
- from this 5 ml suspension of ascospores, some successive dilutions have been inoculated to count the survivors.
IV. Heating treatment

The thermal destruction was done in a hot water bath. We followed the temperature in the baihes with temperature probes which are metal threads. They were connected to a computer (Toshiba T 1000) through an Intab interface Teknik ab AAC-2. A software (AAC-2 Evaluation ver.5.02) allowed us to see the exact temperature directly in the bath as well as in a tube containing 0.4 ml water.

When the experimental temperature was reached, 0.4 ml of ascospores in suspension in sodium chloride were dropped into tubes (10 cm * 1.5 cm) closed with teflon screw plugs to limit the evaporation. Then, the tubes were put into a hot water bath. The temperature probes allowed us to check out that the temperature rise in a tube with 0.4 ml takes about 1 min. So, 1 minute was taken off from the total time of thermal destruction.

The samples were taken out from the bath at different time intervals and put directly into a ice-water bath to be cooled as fast as possible.

V. Method of counting

From the cooled suspension, many successive dilutions (3 to 6) were made in sodium chloride. All the dilutions are incu8lated in DRBC (Dichloran Rose Bengal Chloramphenicol) petridishes. This medium makes them grow with a small diameter thanks to the Rose Bengal, so it is easier to count many moulds on one small petridish (10 to 100). After 48 hours of incubation at 30° or 35°C, we could read the number of moulds for each intervall.

VI. Calculations

As it is said in the introduction, the formula for non-logarithmic curves by ALDERTON and SNELL (1970) (2) has been used. This formula is:

\[
(\text{Log No} - \text{Log N})^2 = k' * t + C
\]

No and N are the original and surviving numbers of ascospores at t, and t.
t is the time of the thermal destruction for a given and constant temperature.
The term k' is the death rate constant and C is a constant close to 0.
a is a term which allows to linearize.

For one, the initial curve: Log N = f (t ) has been plotted because activation and tailing can be observed from this curve.
Then, the rearrangement of the equation and plotting
\[ \log ( \text{Log No} - \log N ) = f ( \log t ) \]
was used to determine the a value which is the reciprocal slope of the plotting curve. In few papers, the a value used is the value calculated for the lower temperature of treatment (KING and al., 1978) (8) and (BAYNE and MICHENER, 1979) (3) but in another paper one a value is calculated for each treatment temperature (KING and HALBROOK, 1987) (9). In this last paper the method is not detailed nevertheless it is detailed in the two first papers, and by KING himself in the first one. So, we used the a value calculated for the least severe treatment to linearize the curves for all temperatures.

Likewise, the plotting curve \[ ( \log \text{No} - \log N )^2 = f ( t ) \]
(curves No 3) was used to read the \(1/k\) value and to get the \(k'\) value which is the slope of the straight line because \[ \log \text{No} - \log N = k' * t + C \] and the C value.

Then, we were able to calculate the different pasteurization values we wanted.

\* \(1/k\) is the time of 90% reduction in viable count at a given temperature. This value is comparable to the usual D value in logarithmic thermal destructions; it could be read for the decrease of one log on the linearized curves No 3;

\* \(3\log\) is the time for 99.9% reduction in viable count at a given temperature. This value is the most representative value for a non-logarithmic thermal destruction because it goes through a longer time and also a larger part of the non straight curve. We can compare the observed \(3\log\) which is read on the curves No 5 \[ \log \text{No} - \log N = f ( t ) \], on 3 Logs, and the experimental \(3\log\) which is calculated with the formula for non-logarithmic curves. Those two values should be close to each other.

Finally, the plotting of the curve \[ \log (1/k) = f (\text{Temperature}) \] has been used to calculate the z value which is the temperature required to reduce the thermal death time tenfold (1 Log on the curves No 4).

This calculation method is borrowed from ALDERTON and SNELL, 1970 (2); KING and al., 1978 (8); BAYNE and MICHENER, 1979 (3); KING and HALBROOK, 1987 (9); and, KING and WHITEHAND, 1990 (10).
RESULTS AND DISCUSSION

I. Harvesting improvement

These different stages of harvesting ascospores were described before in "materials and methods". Nevertheless, some stages conditions were not enough tested and had to be improved.

On a six-weeks-old culture, we tried the last harvesting found out at the end of the preceding work:

- harvesting of the moulds from petridishes in 6-8 ml of Tween 80 because in sodium chloride they had some problems about aggregation;
- soft homogenization this the magnetic stir in ice water bath during 45 min;
- strong homogenization and break of hypha by mixing in the Sorvall mixer at speed 6000 rpm during 2 min;
- filtration on glass whool: the pieces of hypha were taken out from the suspension;
- 3-4 centrifugations to clean the suspension with new Tween 80, each centrifugation was carried out at the speed of 7000 rpm during 15 min;
- let the suspension be almost freezed and put it in the freezed French Pressure Cell;
- the French Pressure Cell with the suspension inside must be put in the fridge at -40°C or less, during at least 2 hours, most of the time it will be one night;
- the suspension must go through the French Pressure Cell under high pressure;
- at this stage, the suspension was correct but full of very little particulates of hypha and conidia spores so we thought that after defreezing from the French Pressure Cell, a microfiltration with 5 μm Millipore filters could let go through all little particulates (parts of hypha, conidia spores..) but not the ascopores of our strain which are bigger than 5 μm and would stay on the filters.
- harvesting of the ascospores from filters in Tween 80;
- last centrifugation to concentrate this suspension and a high initial concentration to use it in the thermal destruction.

During this first harvesting, we checked every stage by examining the suspension under the microscope. The examination after the filtration on glass whool showed a lot of broken ascospores in the suspension. Some hypotheses could be:

- the speed mixing is too high
- the time mixing is too long
- the culture is too old
- it could be several or even all of them.

So, we decided to try with a four-weeks-old culture and a mixing at 4000 rpm speed during 30 seconds.
The examination of the filtrat after microfiltration showed that there was no ascospores but very few little particulates too. This phenomenon was because the cells on the filter became too thick to let go through even the very small particulates, very fast. Therefore, the cells of ascospores must not become too thick until the end of the microfiltration. Likewise, more thick is the lay of cells, more the pressure on ascospores on the filter is high and more difficulties we had to harvest them from the filter in Tween 80. To avoid this we thought about using many filters (6-8) for each harvesting and let go through each filter only a very small quantity of the suspension.

Most of the time to harvest efficiently and separate the ascospores from the many filters, the magnetic speed had to be high enough and so, very often the filters were broken in small pieces. In that case, another filtration on glass whool had to be done once more to clear the suspension from filters.

Those new conditions and way of microfiltration were tried the next harvesting and were found good enough to obtain a quite pure suspension of ascospores. Anyway, the conidia spores and the parts of hyphae left were killed after few minutes of heating treatment.

The harvesting had been done in sodium chloride (0,85\%) because it was the best medium for immobilization in alginate and we wanted to use the same medium than immobilization medium to compare the two experiments values, and because we didn’t have any aggregation problems with it.

The conclusions are shown on figure 4.
four-weeks-old MOULDS HARVESTING
in 6-8 ml of sodium chloride (0.85%)

↓

SOFT HOMOGENIZATION
during 45 min

↓

MIXING
speed 4000 rpm during 30 s

↓

FILTRATION ON GLASS WHOOL

↓

3-4 CENTRIFUGATION AND CLEANING
speed 7000 rpm during 15 min

↓

FRENCH PRESSURE CELL
freezing more than 2 hours and high pressure

↓

MICROFILTRATION
5 μm, 6-8 filters

↓

HARVESTING OF ASCOSPORES FROM FILTERS
in sodium chloride (100 ml)

↓

FILTRATION ON GLASS WHOOL

↓

CENTRIFUGATION AND CONCENTRATION

figure 4.
II. Heat-resistance on free ascospores

To be able to use the results with the formula we detailed, the initial concentration must be high enough to get a destruction of at least 4 Logs on the curve. That means the initial concentration must be higher than $10^6$ ascospores/ml.

a) Results

The first heat-treatment has tested 80°C and 85°C. The results are:

\[
\text{Curve No 1.1} \\
\log N = f(t)
\]
Curve No 2.1
\[ \log (\log N) = f (\log t) \]
\[ y = 3.339676 + 2.1098632x \quad r = 0.984 \]

Log (\log N)

Log (Time in min)

-- Temp 80°C
-- Temp 85°C

Curve No 3.1
\[ (\log N) \exp (0.47) = k' \cdot t + C \]
\[ (\log N) \exp (0.47) \]

Time in min

-- Temp 80°C
-- Temp 85°C

Curve No 4.1
\[ \log (1/k) = f (\text{Temperature}) \]

Log (1/k)

Temperature in °C
As it is shown on the curve No 2.1, the slope is 2.11 which is \(a^{-1}\). So, we could calculate than\(a = 0.47\), with a correlation \(r=0.984\).

The curves No 3 allowed us to read the \((1/k)\) value on one log decrease (Appendix I.) and we found:
- \((1/k)_{80^\circ} = 39\text{ min}\) with a correlation on this straight curve \(r=0.990\).
- \((1/k)_{82.5^\circ} = 10\text{ min}\) with a correlation on this straight curve \(r=0.946\).

The straight curve No 4.1 allowed us to measure the "z" value (Appendix I.). We found \(z = 8.3^\circ \text{C}\).

Then, we had to read on the curves No 5.1 (Appendix I.), the \((3\log)_{obs}\) value and we had to compare it to the \((3\log)_{exp}\) calculated by the experimental formula.

\[
\begin{align*}
(3\log_{80^\circ})_{obs} & = 7.5\text{ min} & (3\log_{88^\circ})_{exp} & = 13.2\text{ min} \\
(3\log_{82.5^\circ})_{obs} & = 51\text{ min} & (3\log_{88^\circ})_{exp} & = 67.4\text{ min}
\end{align*}
\]

The second heat-treatment has tested \(80^\circ \text{C}\) and \(82.5^\circ \text{C}\). The results are:
Curve No 2.2
\[ \log (\log N - \log N) = f(t) \]
\[ y = -1.437315 + 1.2007283x, \quad r = 0.970 \]

\[ \log (\log N - \log N) \]

Temp 80°C

Temp 82.5°C

Log (Time in min)

Curve No 3.2
\[ (\log N - \log N) \exp(0.833) = k't + c \]

\[ (\log N - \log N) \exp(0.833) \]

Temp 80°C

Temp 82.5°C

Time in min

Curve No 4.2
\[ \log \left( \frac{1}{k} \right) = f(\text{Temperature}) \]

\[ \log \left( \frac{1}{k} \right) \]

76 78 80 82 84 86 88 90

Temperature in °C
On curve No 2.2, the slope is 1.2. So, $a = (1.2)^{-1} = 0.833$, with a correlation $r=0.970$.

The reading of one log decrease on the curves No 3.2 (Appendix II.) gave us the $(1/k)$ values which are:

- $(1/k)_{80^\circ}$ = 17 min with a correlation on this straight curve $r=0.987$.
- $(1/k)_{82.5^\circ}$ = 9 min with a correlation on this straight curve $r=0.992$.

On the curve No 4.2, the "z" value has been measured and $z = 9^\circ$C (Appendix II.) is found.

Then, the curve No 5.2 (Appendix II.) allowed us to calculate and compare the two values $(3\log)_{obs}$ and $(3\log)_{cal}$ calculated with the experimental formula:

- $(3\log)_{80^\circ})_{obs} = 36.5$ min
- $(3\log)_{82.5^\circ})_{obs} = 16.5$ min
- $(3\log)_{80^\circ})_{exp} = 40.6$ min
- $(3\log)_{82.5^\circ})_{exp} = 19.04$ min

The third heat treatment has tested $82.5^\circ$C and $85^\circ$C. The results are:

Curve No 1.3
Log N = f (t)
Curve No 2.3
\log (\log N - \log N) = f (\log t)
y = -1.608106 + 1.5463245x r=0.978
\log (\log N - \log N)

Log (Time in min)

---
T° 82,5°C
Temp 85°C

Curve No 3.3
(\log N - \log N) \exp (0.647) = k't + c
(\log N - \log N) \exp (0.647)

Time in min

---
T° 82,5°C
Temp 85°C

Curve No 4.3
\log (1/k) = f (Temperature)

Log (1/k)

---
Temperature in °C

17
♦ On the curve No 2,3, a = 0.647 has been found with a correlation r=0.978.

♦ On the curves No 3,3 (Appendix III.), the (1/K) values were read:
  (1/k)<sub>32,5</sub> = 12.9 min with a correlation on this straight curve r=0.981,
  (1/k)<sub>50</sub> = 7.8 min With a correlation on this straight curve r=0.981.

♦ z = 11.4°C was found on the curves No 4,3 (Appendix III.).

♦ Then, the values (3Log)<sub>obs</sub> read on the curves No 5,3 (Appendix III.) have been compared to the (3Log)<sub>exp</sub> values calculated by the modified formula:

\[
\begin{align*}
(3\text{Log}_{82.5})_{\text{obs}} &= 23.5 \text{ min} \\
(3\text{Log}_{88})_{\text{obs}} &= 11.6 \text{ min} \\
(3\text{Log}_{82.5})_{\text{exp}} &= 23.6 \text{ min} \\
(3\text{Log}_{88})_{\text{exp}} &= 11.9 \text{ min}
\end{align*}
\]

b) Discussion

The first treatment had been done without known data. So, we can see that we didn’t have enough samples on the heat-destruction curve. Then, for the second and the third heat-treatment some samples have been taken at the beginning of the heat-treatment to be able to see the activation on the curve. The problem is that we can’t be sure that the No in the first heat-treatment is the highest concentration because we cannot see any activation on it. That is maybe one of the reasons why there is so much difference between the two values (3Log)<sub>obs</sub> and (3Log)<sub>exp</sub>. It is important to choose the No as the highest concentration and that is why it was important to study activation for lower temperatures.

c) Activation

Even in the second heat-treatment we couldn’t be sure that we got the highest concentration as possible because we had found an activation at 80°C, but 80°C is a high temperature for T. flavus. Maybe an activation at a lower temperature could give a higher concentration than for 80°C. To check it, we tried an activation with the same suspension at 70°C, 75°C, 80°C and 85°C. The results are shown on the curve called "Activation".

The activation exists but it is a very small one. To compare our activation with a real activation, some works had been done about the activation of Byssoschlamys fulva and this fungus has an activation at 80°C from 10<sup>3</sup> ascospores/ml to 10<sup>6</sup> ascospores/ml. So, Talaromyces flavus has not a big enough activation to change anything in our method. No activation had been decided before the heat-treatment after that study.
III. Heat-resistance on immobilized ascospores

a) Results

The first heat-treatment tested 80°C and 85°C. The shape of the heat-destruction is:

\[ \text{Curve No 1.4} \]
\[ \log N = f(t) \]
Curve No 2.4
\[ \log (\log N - \log N_0) = f(\log t) \]
\[ y = -1.644 + 1.005 \times x, \quad r = 0.972 \]

Log (Time in min)

Curve No 3.4
\[ (\log N - \log N_0) \exp(0.946) = k't + C \]

Time in min

Curve No 4.4
\[ \log (1/k) = f(Temperature) \]
♦ On the curve No 2.4, we found $a = 0.946$ with a correlation $r=0.972$.

♦ The curves No 3.4 allowed us to find those $(1/k)$ values (Appendix IV.):

$(1/k)_{29^\text{min}} = 29\, \text{min}$ with a correlation on this straight curve $r=0.956$,
$(1/k)_{5^\text{min}} = 5\, \text{min}$ with a correlation on this straight curve $r=0.992$.

♦ $z = 6.5^\circ\text{C}$ was read on the curve No 4.4 (Appendix IV.),

♦ Then, on the curves No 5.4 were read the $(3\log)_\text{obs}$ values (Appendix IV.) and the $(3\log)_\text{exp}$ were calculated from the modified formula:

$(3\log)_{29^\text{min}} = 76\, \text{min}$ \hspace{1cm} $(3\log)_{83^\text{min}} = 83.8\, \text{min}$

$(3\log)_{5^\text{min}} = 11\, \text{min}$ \hspace{1cm} $(3\log)_{15.65^\text{min}}$

The second heat-treatment has tested $80^\circ\text{C}$ and $82.5^\circ\text{C}$. The results are:

Curve No 1.5
$\log N = f(t)$

![Graph of $\log N$ vs. Time in min]

- Temp $80^\circ\text{C}$
- To $82.5^\circ\text{C}$

21
Curve No 2.5
\[ \log (\log N_0 - \log N) = f(\log t) \]
\[ y = -0.87641 + 0.476514x \quad r = 0.912 \]

Curve No 3.5
\[ (\log N_0 - \log N) \exp(2,1) = k't + C \]

Temperature:
- Temp 80°C
- Temp 82.5°C
The same method was used, so here are the values found:

- $a = 2.1$ on the curve No 2.5 with a correlation $r=0.912$;

- Curves No 3.5 (Appendix V.):
  
  - $(1/k)_{42.5} = 53$ min with a correlation on this straight curve $r=0.932$.
  - $(1/k)_{50} = 49$ min with a correlation on this straight curve $r=0.927$.

We can see that it is something wrong with this heat-treatment so it is no use to go further in the calculations.

The last heat-treatment tested 82.5°C and 85°C and the results are:
Curve No 2.6
\[
\log (\log N - \log N) = f(\log t)
\]
y = -0.95987 + 0.805091x, r = 0.921

Log (Time in min)

Curve No 3.6
\[
(\log N - \log N) \exp (1.24) = k't + C
\]
\[
(\log N - \log N) \exp (1.24)
\]

Time in min

Curve No 4.6
\[
\log (1/k) = f(\text{Temperature})
\]

Log (1/k)

Temperature in °C
a = 1.24 on the curve No 2.6 with a correlation r=0.921.

Curves No 3.6 (Appendix VI.):

\[(1/k)_{32,5^\circ} = 17 \text{ min} \] with a correlation on this straight curve r=0.948,

\[(1/k)_{85^\circ} = 9 \text{ min} \] with a correlation on this straight curve r=0.966.

The z value is \( z = 8.8^\circ \text{C} \) on the curve No 4.6 (Appendix VI.).

Curves No 5.6 (Appendix VI.):

\[(3\text{Log}_{82,5^\circ})_{\text{obs}} = 61 \text{ min} \]

\[(3\text{Log}_{82,5^\circ})_{\text{exp}} = 68.53 \text{ min} \]

\[(3\text{Log}_{85^\circ})_{\text{obs}} = 31 \text{ min} \]

\[(3\text{Log}_{85^\circ})_{\text{exp}} = 29.5 \text{ min} \]

b) discussion

The very bad second heat-treatment has no obvious explanation. Those results are really missing to compare values to each other for a same temperature. The reason of this spoiled heat-treatment could be something wrong in the harvesting (contamination, broken ascospores…). The temperature probes allow us to be almost sure that the heat-treatment had been done at the right temperature.
### IV. General discussion

<table>
<thead>
<tr>
<th></th>
<th>Free</th>
<th>Immobiliz</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1/k)</td>
<td>(3Log)\text{obs}</td>
</tr>
<tr>
<td><strong>80°C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in min</td>
<td>39</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>36.5</td>
</tr>
<tr>
<td><strong>82.5°C</strong></td>
<td>9</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>12.9</td>
<td>23.5</td>
</tr>
<tr>
<td><strong>85°C</strong></td>
<td>10</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>7.8</td>
<td>11.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>First treatment (80°C &amp; 85°C)</th>
<th>Second Treatment (80°C &amp; 82.5°C)</th>
<th>Third treatment (82.5°C &amp; 85°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a</strong></td>
<td>0.47 (r=0.984)</td>
<td>0.946 (r=0.972)</td>
<td>0.833 (r=0.97)</td>
</tr>
<tr>
<td><strong>z</strong></td>
<td>8.3°C</td>
<td>6.5°C</td>
<td>9°C</td>
</tr>
</tbody>
</table>
The results show that the values are not really reproducible. Even the method had been always as much as possible the same, the biology makes some difference from batch to batch.

The \((1/k)\) values are rather different for the temperature, at least for free ascospores because it is difficult to say for immobilized ascospores (one heat-treatment is missing). The \((3\log)_{obs}\) value and the \((3\log)_{exp}\) value are always in the same order but some are very close to each other (11.6 min and 11.9 min) and some are much more different from each other (51 min and 67.3 min).

The correlations are correct (more than 0.95 most of them). That means that the \(a\) factor is a good value to linearize the non-logarithmic heat-destruction curves. The method seems to be well adapted to this kind of heat-destruction curves.

Not enough results had been collected to surely conclude but we can outline the tendencies of that study.

We can find out two mains points between free and immobilized ascospores:

- The \(a\) value seems to be lower for immobilized ascospores than for free ascospores. This means immobilized ascospores are less heat-resistant at the beginning of the heat-treatment but more heat-resistant at the end of the treatment than free ascospores. The form of the curve \(\log N = f(t)\) is also different. The curve for free ascospores has a big sholder but the curve for the immobilized ascospores has a little sholder but almost a tailing at the end.

- The \(z\) value seems to be lower for immobilized ascospores than for free ascospores. This means immobilized ascospores are less heat-resistant on one \(\log\) than free ascospores.

Many reasons are possible for that: the alginate could protect the ascospores against a long heating time, the ascospores at the surface could be destroyed faster than free ascospores and that could be why the first \(\log\) is faster to be destroyed but the next ones slower than for free ascospores destruction.

The values found in that study are comparable to the literature values. KING and HALBROOK, 1987 (9) found a \(a\) value between 0.202 and 0.7258 for different ascospores strains, and we found a range of \(a\) value for free ascospores between 0.47 and 0.833. The \((1/k)\) values found at 80°C in our study (39 and 17 min) are lower than the ones found by KING and WHITEHAND, 1990 (10) (191 min) or by KING and HALBROOK, 1987 (9) (63.96 and 113 min). So, our strain seems to be less heat-resistant than the ones they used.
CONCLUSION

The results of that study show we can hope to immobilize *Talaromyces flavus* ascospores as bioindicators. This specie produces mostly ascospores which are easy to harvest because they are not too fragile. However, it is a lot of problems left after this study.

We have to check the repeatability of the specie on a high number of experiments. Two experiments for temperature is really too less to conclude. Then, if the values are reproducible, the reasons of the difference of heat-resistance between free and immobilized ascospores have to be found out. It could be because the ascospores start to germinate in the alginate even in one hour. It could be another change in the biology of ascospores. But whatever it is, it has to be reproducible.

The last problem to check will be the storage. If it is possible to storage free ascospores or even better, immobilized ascospores, the best way of storage has to be found out.
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APPENDIX I.
Curve No 3.1

\[( \log \text{No} - \log \text{N} ) \exp(0.47) = k't + C \]

\[( \log \text{No} - \log \text{N} ) \exp(0.47) \]
Curve No 4.1

\[ \log \left( \frac{1}{k} \right) = f \left( \text{Temperature} \right) \]
APPENDIX II.
Curve No 3.2

\((\text{Log No} - \text{Log N}) \exp (0.833) = k't + C\)

\((\text{Log No} - \text{Log N}) \exp (0.833)\)
Curve No 4.2

\[ \log \left( \frac{1}{k} \right) = f (\text{Temperature}) \]
Curve No 5.2

\[
\log \text{No} - \log N = f(t)
\]
APPENDIX III.
Curve No 5.3

\[(\log N_0 - \log N) = f(t)\]

-0.66 min (80°C) to 63.5 min (85.5°C)

Time in min

Temp 82.5°C
APPENDIX IV.
Curve No 3.4

\[(\text{Log No} - \text{Log N}) \exp(0.946) = k't + C\]

\[(\text{Log No} - \text{Log N}) \exp(0.946)\]

Time in min

- Temp 80°C
- Temp 85°C
APPENDIX V.
Curve No 3.5

\[(\text{Log No} - \text{Log N}) \exp (2,1) = k't + C\]

\[(\log \text{No} - \log \text{N}) \exp (2,1)\]
APPENDIX VI.
Curve No 4.6

\[ \log \left( \frac{1}{k} \right) = f(\text{Temperature}) \]
Curve No 5.6
(Log No - Log N) = f(t)