Fungal Spoilages in "Hot Fill and Inversion" Beverages and Juices

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Presentation Outline

1. Types of fungi, their forms of existence, as well as their heat resistance
2. Fungal dispersal & airborne mold
3. Mechanisms for fungal survival/stress resistance /Factors affecting fungal heat resistance
4. How to control spoilages by fungi (focusing on HRM)
5. Effectiveness of “Inversion”
6. On-going monitoring of fungal spoilage

Fungal spoilage is a reality

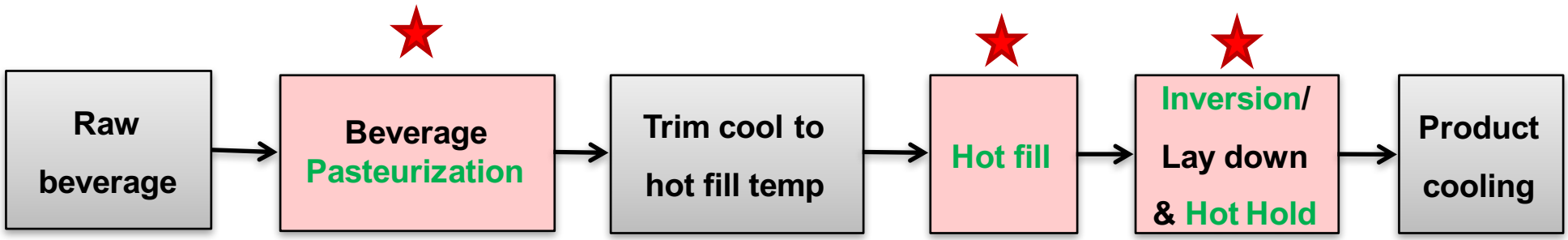
Table 1

Incidence of bacterial and fungal spoilage and intervention strategies used in the production of fruit and vegetable juices. Results are reported as the percent of total respondents, excluding answers of "N/A" for product specific questions.

Survey Question	"Yes" Responses	"No" Responses
Is *Alicyclobacillus* contamination a concern for your company?	78%	22%
Have you ever experienced heat resistant mold in your finished product?	64%	36%
Do you utilize ingredients or finished product testing for heat resistant molds?	75%	25%
If you manufacture apple juice products, have you had to discard ingredients or finished product to control patulin?	67%	33%
Have you had to discard ingredients or product due to spoilage in the past year?	69%	31%

Synder, A.B. and R. W. Worobo. 2018. The incidence and impact of microbial spoilage in the production of fruit and vegetable juices as reported by juice manufacturers. Food Control 85:144-150

"Hot Fill and Inversion" - Definition



- **Hot Fill & Inversion** (scope of presentation):
  - Fill **pasteurized** (usually 85-95C for 15-60 sec) products **hot** (e.g., 80-95C) into empty packages and seal them.
  - **Invert** (or lay down) (e.g., 1 to 30 sec) and then **hot hold** the packages for a short time (e.g., 30-120 sec) to inactivate the spoilage organisms that come from the bottle/caps or filling contamination, prior to cooling.

Presentation

Types of fungi, their forms of existence & heat resistance

Types of fungi / their forms of existence

Fungi important to **beverage spoilage**:

- **Zygomycetes** (Class), e.g., *Mucor*, *Rhizopus* — *"zygo"* = "joining" or "yoke"
- **Ascomycetes**, e.g., *Saccharomyces cerevisiae*, *Talaromyces* sp., *Neosartorya* sp., *Byssochlamys sp.* — *"asco"* = "sac"
- **Deuteromycetes** (nonasexual /imperfect fungi, e.g., *Geotrichum* sp.

Anamorph	+	**Teleomorph**	=	**Holomorph**
Asexual		Sexual		Whole fungus
Imperfect fungus				**Perfect** fungus

Heat Resistant Mold (HRM)

Types of fungi / their forms of existence

One fungus may assume different forms of existence, which have:

- Different physiological states
- Different resistance to stresses (e.g., heat)

Types of fungi / their forms of existence

Zygomycetes

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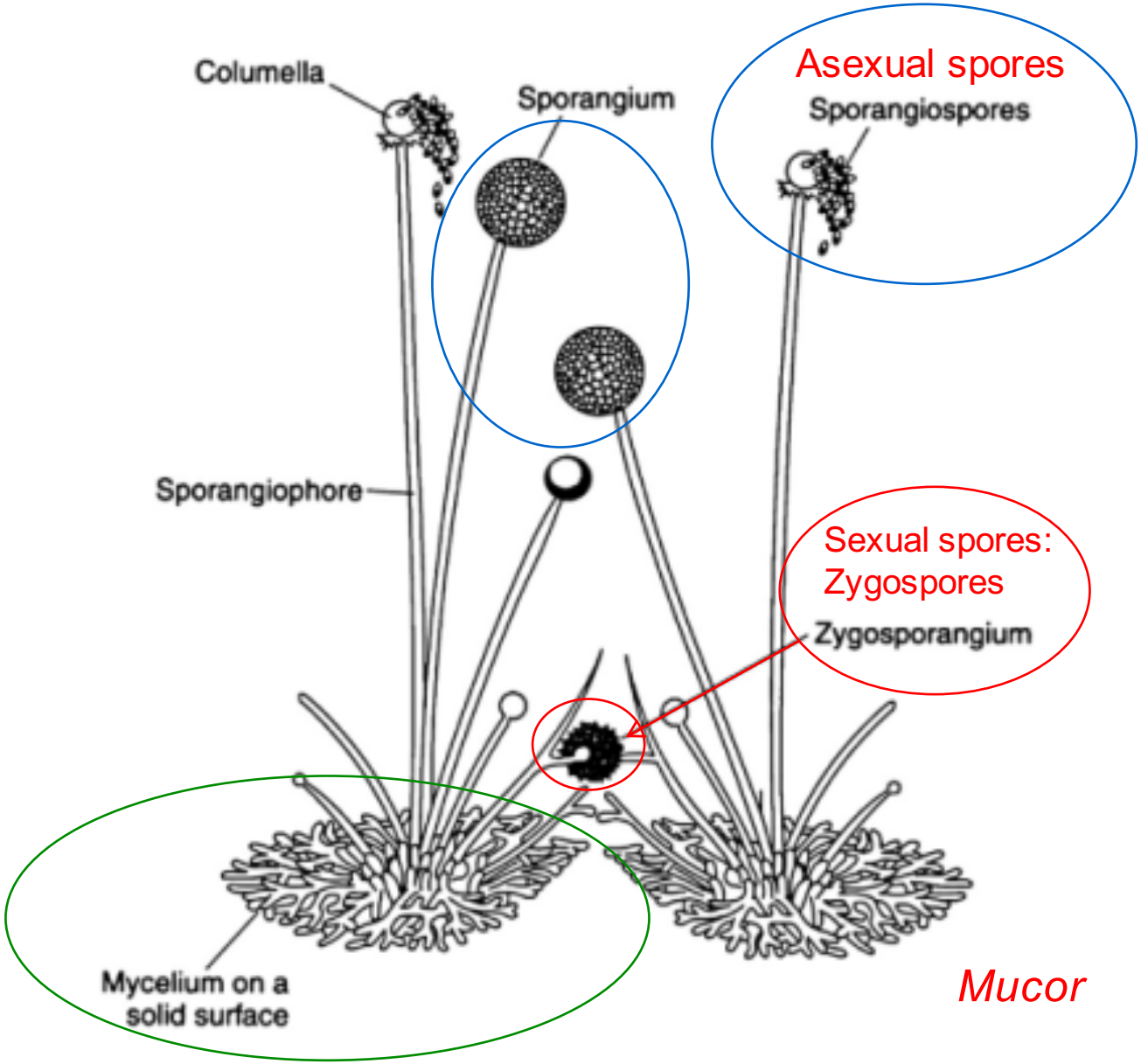
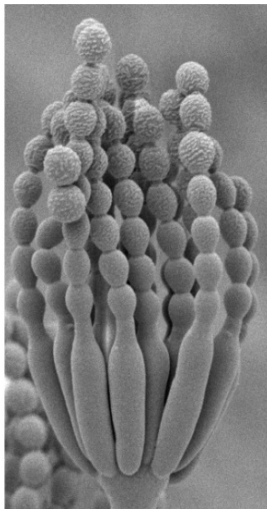


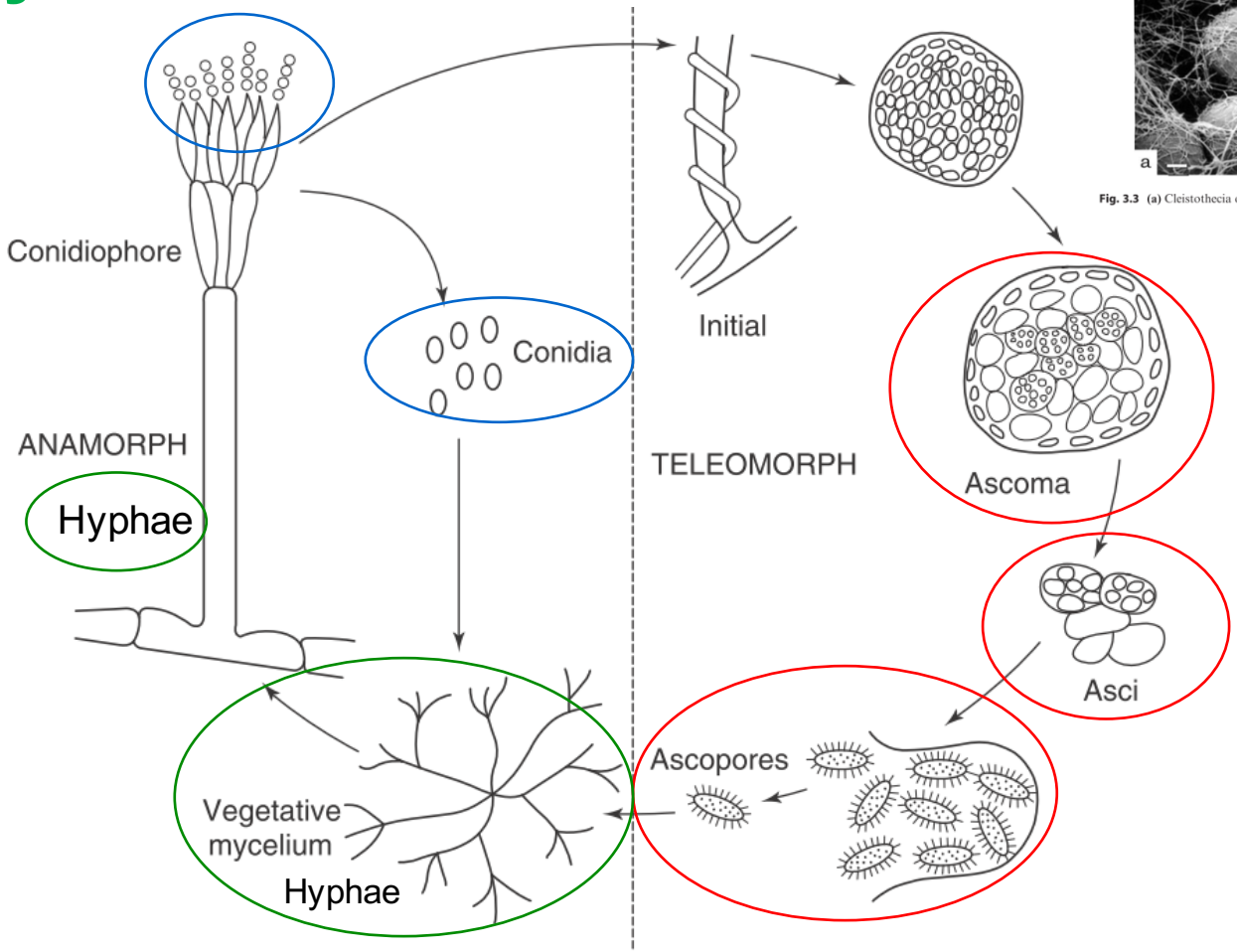
Mucor

Source of graph:
Botha, A., & A. Botes. 2014. *Mucor.* Encyclopedia of Food Microbiology, 2nd ed. p834-840

Types of fungi / their forms of existence

Ascomycetes





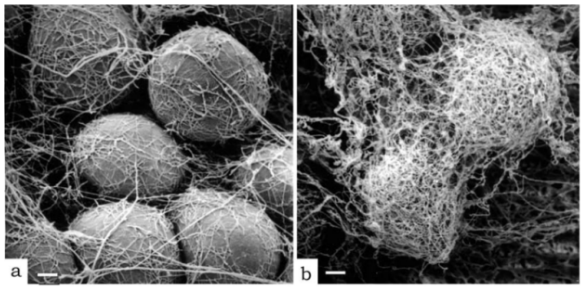


Fig. 3.3 (a) Cleistothecia of *Eupenicillium*; (b) gymnothecia of *Talaromyces*. SEM. Bars = 50 µm

***Neosartorya fischeri*:**

- **Ascoma** (cleistothecium): 400 um diameter
- **Ascospore**: 5.5-7.0 x 3.5-4.5 um
- **Conidia**: 2.5 – 3.0 um long (subspheroidal or ellipsoidal)
- **Aerial mycelia**: 300 – 500 um long

Figure 1 Ascomycete–Deuteromycete relationship showing anamorphic and teleomorphic stages.

Anamorph	+	**Teleomorph**	=	**Holomorph**
Asexual Imperfect fungi		Sexual		Whole fungus Perfect fungus

- Cousin, M.A. 2014. Classification of the Eukaryotic Ascomycetes. Encyclopedia of Food Microbiology. 2nd ed.
- Tournas, V. 1994. Heat-Resistant Fungi of Importance to the Food and Beverage Industry. Crit. Rev. Microbiol. 20:243-263

One fungus has different forms of existence

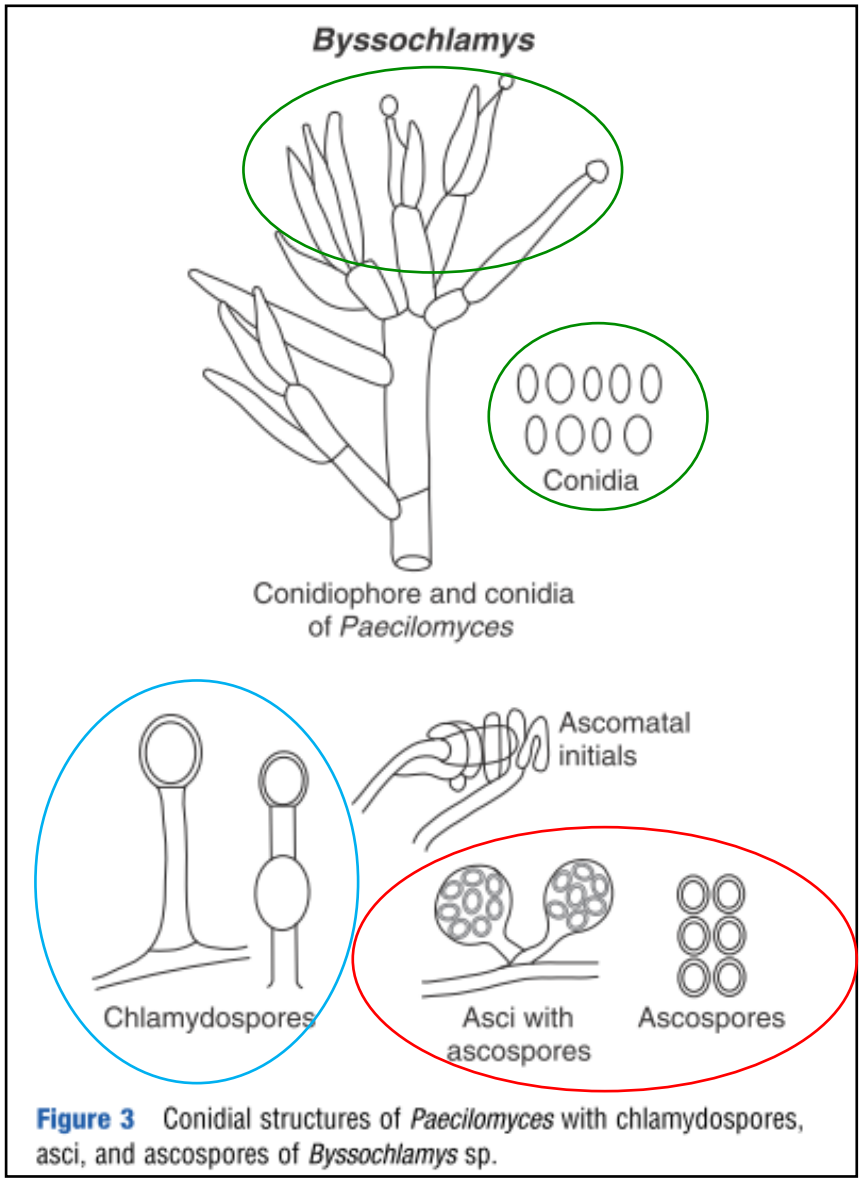


Figure 3 Conidial structures of *Paecilomyces* with chlamydospores, asci, and ascospores of *Byssochlamys* sp.

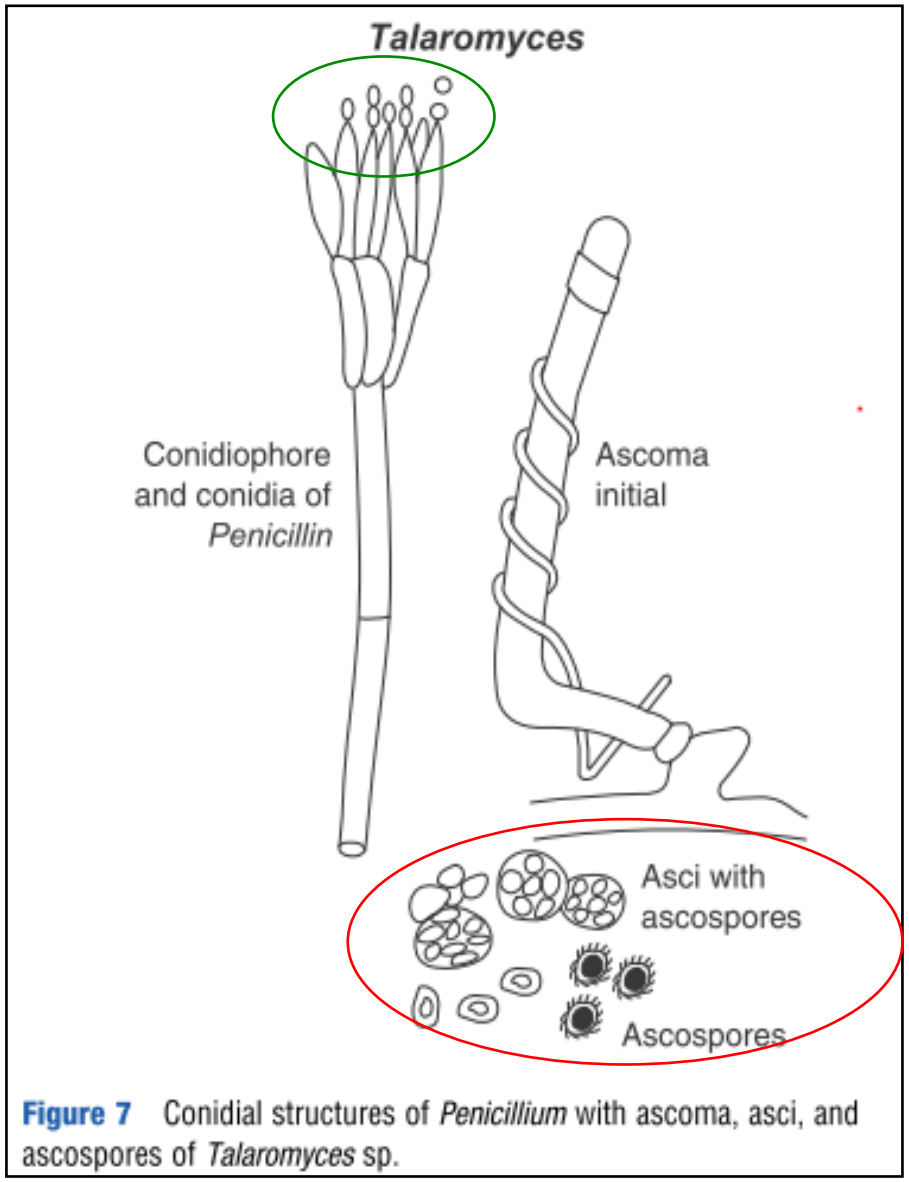


Figure 7 Conidial structures of *Penicillium* with ascoma, asci, and ascospores of *Talaromyces* sp.

Cousin, M.A. 2014. Classification of the Eukaryotic Ascomycetes. Encyclopedia of Food Microbiology. 2nd ed.

Types of fungi / their forms of existence

- **Ascomycetes**

Spore-forming structures (conidiophores) of an *Aspergillus* species (*Eurotium*) originating from cured ham as observed by high-resolution stereomicroscopy. Rows of single-celled conidia are visible on the conidiophore.

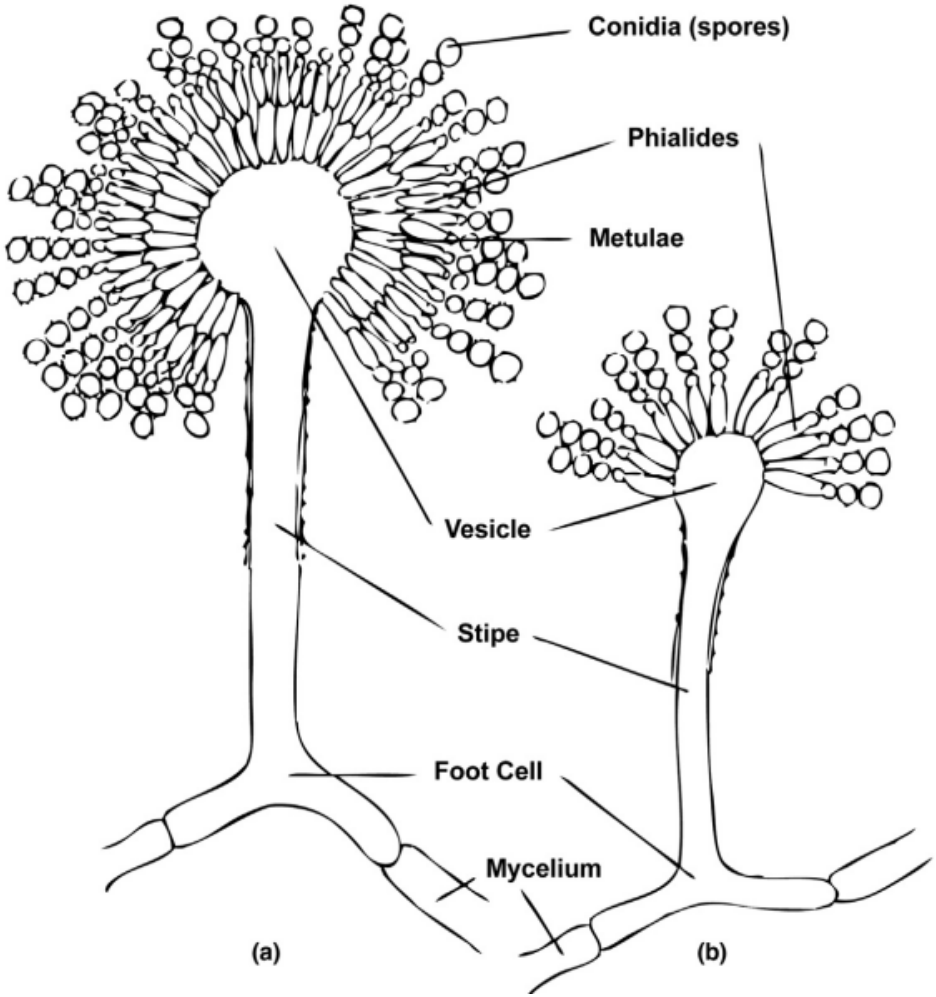
Aspergillus

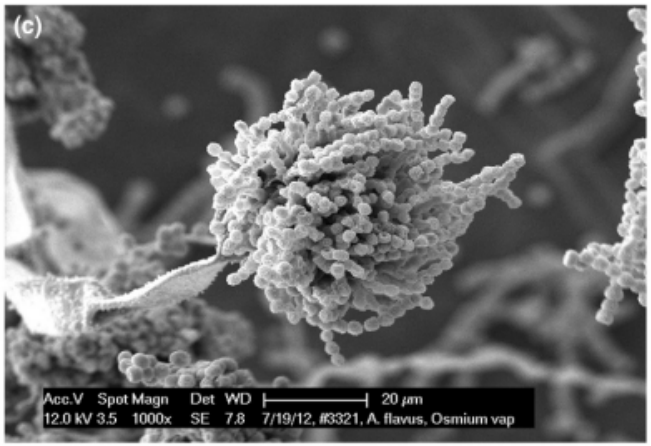
- Produce enormous # of conidia: >10,000 conidia /conidiophore
- These asexual spores are among the most dominant fungal structures in the air – More than 10 *A. fumigatus* spores/m^3 found in outdoor air

1. Dijksterhuis, J. 2017. The fungal spore and food spoilage. Current Opinion in Food Science 17:68-74
2. Teertstra, W.r., M. Tegelaar, J. Dijksterhuis, E. A. Golovina, R.A. Ohm, & H.A.B. Wosten. 2017. Maturation of conidia on conidiophores of *Aspergillus niger*. Fungal Genetics and Biology. 98:61-70

Types of fungi / their forms of existence

- **Ascomycetes**





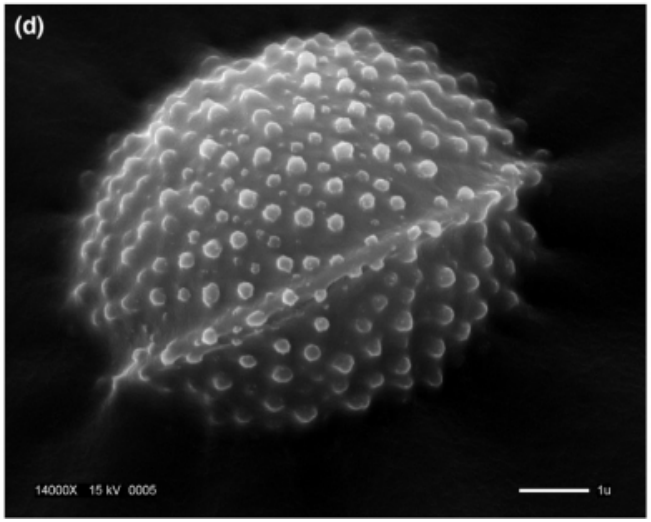
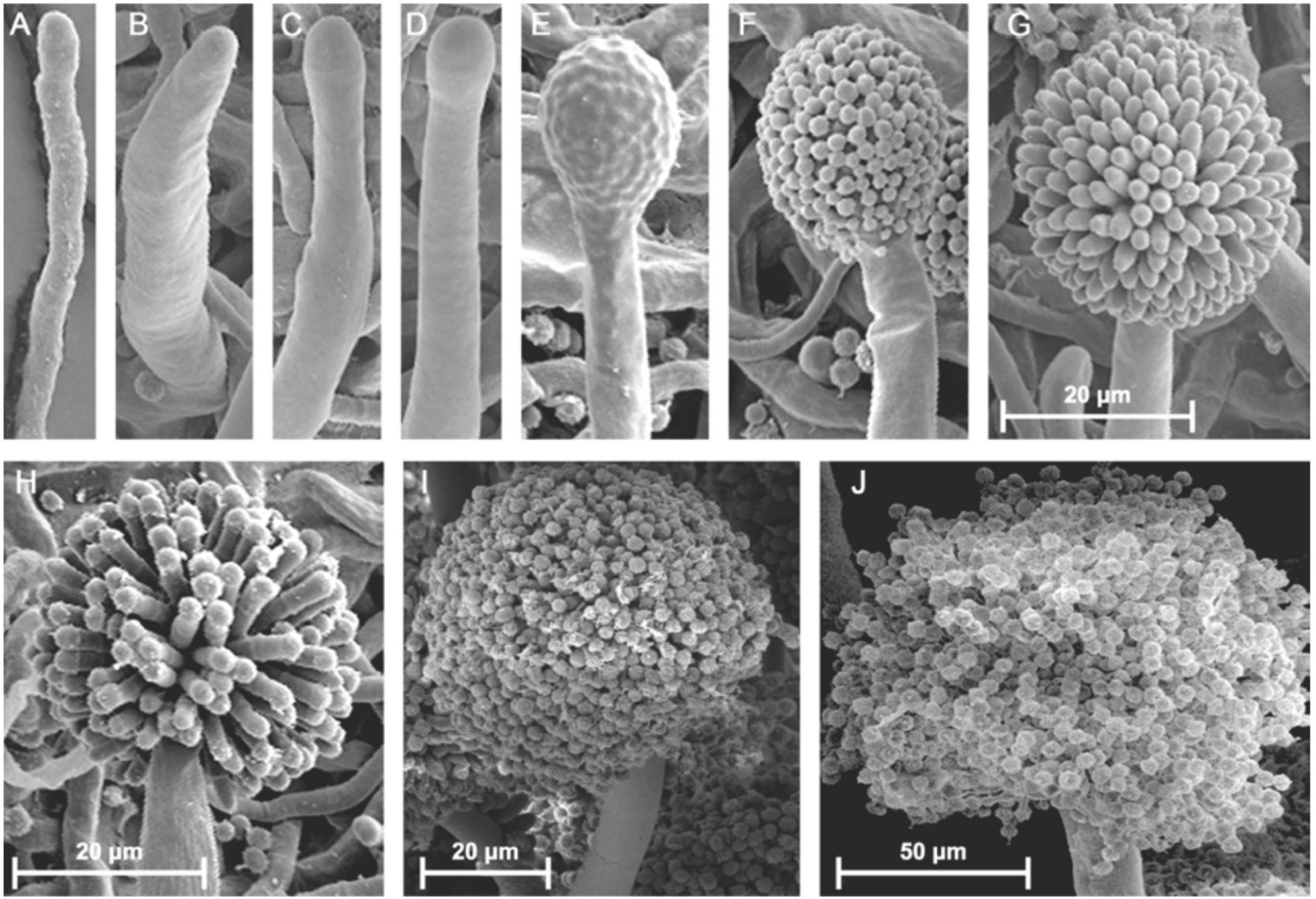


Figure 2 The conidiophore is attached to the mycelium by a characteristically foot-shaped structure. Conidial heads, (a) biseriate and (b) uniseriate, are characteristic of *Aspergillus flavus*. Electron micrographs of *A. flavus*: (c) Conidiophore (magnification × 1000), (d) Photomicrograph of an ascospore from *Petromyces flavus*.

Bhatnagar, D., K.C. Ehrlich, and G.G. Moore. 2014. *Aspergillus flavus*. Encyclopedia of Food Microbiology, 2nd ed. p.83-91

Types of fungi / their forms of existence

Aspergillus niger - different stages during growth



Source of graph: Krijgsheld et al. 2012. Development in *Aspergillus*. Studies in Mycology 74:1-29

Fig. 3. Development of *A. niger* monitored by scanning electron microscopy. The vegetative mycelium forms two types of aerial hyphae. One type is similar to vegetative hyphae (A), while the other type is 2–3 times thicker (B). The tips of the latter aerial hyphae may swell to form a vesicle (C,D). Buds are formed on the vesicle (E) that develop into metulae (F, G). Phialides are formed on top of the metulae (H), which give rise to chains of conidia (I, J). The bar in G also holds for A–F.

Types of fungi / their forms of existence

Deuteromycetes

(nonsexual /imperfect fungi)

Geotrichum - "machinery mold"

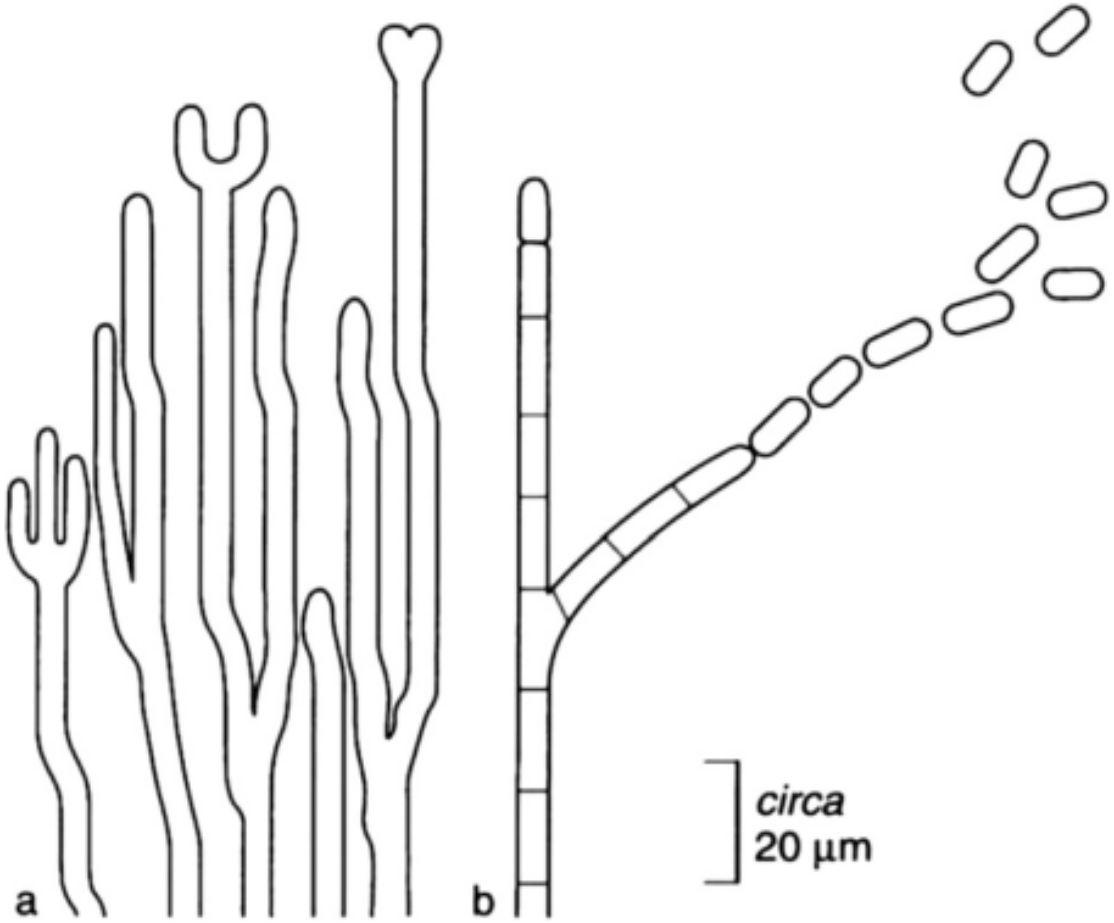
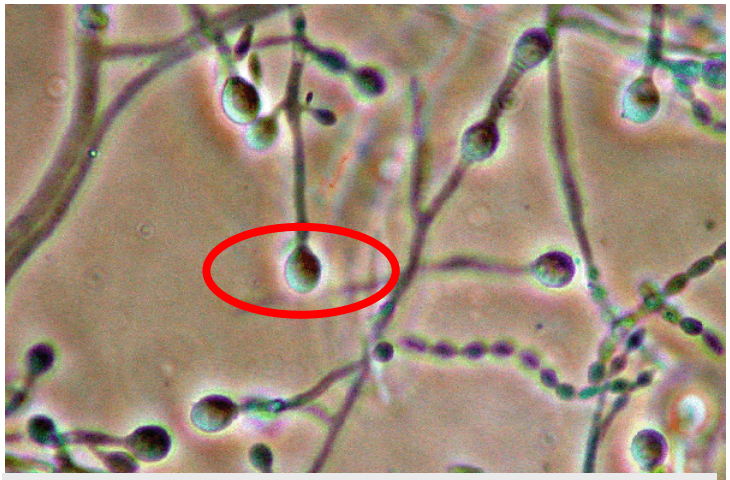


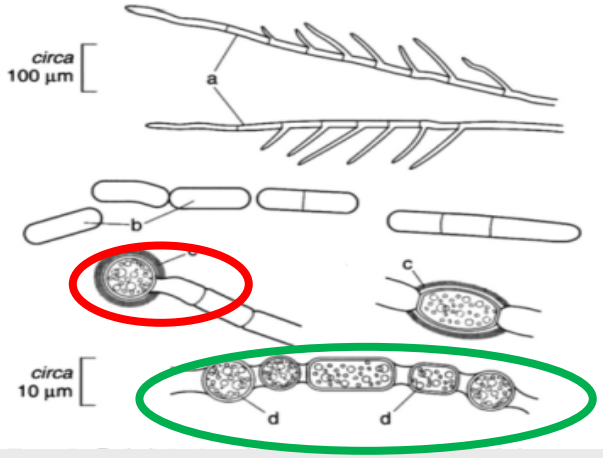
Figure 4 Typical structures formed by *Geotrichum candidum* (*Galactomyces candidus*) on general purpose media: (a) expanding hyphae during active growth; and (b) older hyphae tend to break up into arthric conidia.

Source of graph:
Botha, A. & A. Botes. 2014. *Geotrichum*. Encyclopedia of Food Microbiology, 2nd ed. p88-93

One fungus may have different forms of existence



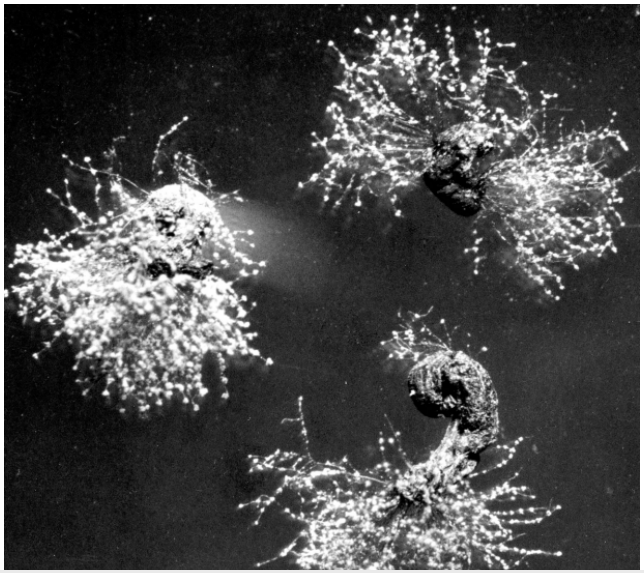
Chlamydospore - *Paecilomyces*



Chlamydospore & endospores – *Geotrichum fragrans*

Chlamydospore:

- Thick-walled, big resting spore, from asexual reproduction or sexual reproduction (rare)



Sclerotia (germinating) – *Botrytis cinerea*

Sclerotia:

- Hard compacted mass of mycelium

Source of *B. cinerea* graph:
Jackson, R.S. 2014. *Botrytis*. Encyclopedia of Food Microbiology, 2nd ed. p288-296

Different forms of mold have different heat resistance

Note: D-values & log reductions given here – may not be accurate - just for illustrative purpose

Mold Asco-spores
D_{90C}: 1 - 12 min; z = 6-7C

Mold Asci

Mold Ascomata
(cleistothecia, gymnothecia, etc.)

Sclerotia
(?)

Zygospores
(?)

Chlamydospores (?)

Conidia / Sporangiospores
D_{60C}: 0.01- 0.5 min

Mold Mycelia
D_{60C}: 0.001 min

Yeast ascospores
D_{60C}: up to ~20 min

Yeast vegetative cells
D_{60C}: 0.001 – 0.005 min

Heat Resistance

160F (71.11C)/6 sec Kills:
(only for illustrating relative heat resistance)

Mold Ascospores
0 log

"Fill hot & then Invert"– unlikely to kill HRM

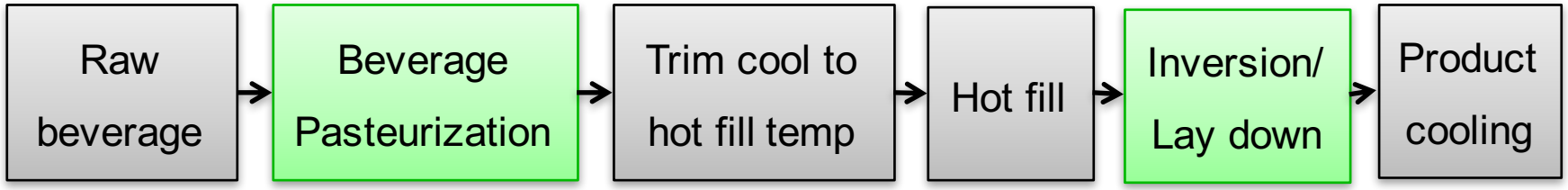
Conidia / Sporangiospores
ca.5 to >1000 logs

Mold Mycelia
>900 logs

Yeast ascospores
0.4 – ca. 3 log

Yeast vegetative cells
2 to >1000 logs

Spoilage organisms of concern in "Hot Fill & Inversion"



- **Pathogens**, e.g.,
  - *E. coli O157:H7*
  - Viruses
  - Protozoa
- **Spoilage bacteria**:
  - *B. coagulans*
  - Butyric anaerobes
  - *Alicyclobacillus*
  - Lactic acid bacteria, etc.
- **Yeast**
  - Vegetative cells
  - Ascospores
- **Mold**
  - Hyphae
  - Conidia /sporangiospores
  - Ascospores
  - Asci
  - Ascomata

Package bioload & filling contamination

Cooling contamination

"+" = possible presence/survival/contamination

Heat resistance – different forms of fungal structures

- **Yeast vegetative cells vs ascospores**
  - D_{60C} of *Saccharomyces* spp. ascospores were 50-150 fold higher than that of the corresponding vegetative cells (in 0.05M citrate-phosphate buffer pH 4.5) (1)

• Ascospores	D_{60C}=1.5 - 22.5 min (z-value = 4.0 - 6.5C)
• Vegetative cells	D_{60C} = <0.1- 0.32 min

  - Some strains of *S. cerevisiae* ascospores had the highest heat resistance among yeast (even among nonsexual mold forms)

160F (71.11C) /6 sec
Inactivates:

- 0.5 -3.4 log
- >64 log

1. Put, H.M.C. & J. De Jong. 1982. The heat resistance of ascospores of four *Saccharomyces* spp. isolated from spoiled heat-processed soft drinks and fruit products. J. Appl. Bacteriol. 52:235-243

Heat resistance – different forms of fungal structures

- **Vegetative cells** of yeasts - isolated from spoiled acid/acidied products (1)
  - Spoilage *Sacchromyces cevesiae* **vegetative cells** were most heat-resistant among the spoilage yeasts, mold (conidia), and lactic acid bacteria tested

Yeast culture preparation:
"Yeast were grown on Sabouraud dextrose agar for no longer than 4 days at 30C. Vegetative cells were harvested from Sabouraud dextrose agar with phosphate-buffered saline pH 7"

Were some of the vegetative cells ascospores?

TABLE 1. *Heat resistance of* Saccharomyces cerevisiae *in juice products*[a]

Product	pH	*D*-value (min) at temp (°C/°F) 57/135	60/140	63/145	*z*-value (°C/°F)
Tomato	4.5	15 ± 0.5	3.9 ± 0.3	1.1 ± 0.4	5.2/9.4
Tomato	4.2	16 ± 0.5	4.1 ± 1.2	0.64 ± 0.1	4.3/7.7
Grapefruit juice	3.3	9.3 ± 2.1	2.8 ± 0.9	0.98 ± 0.6	6.1/11
Apple juice	3.9	9.1 ± 0.5	2.1 ± 0.2	0.3 ± 0.1	4.0/7.2
Apple juice	3.5	13	3.1	0.6	6.0/11
Calcium-fortified apple juice	3.9	32	6.9	2.1	5.1/9.2
Juice product	2.8	9.4	2.8	0.4	5.8/11

[a] Values with standard deviations are averages of three independent experiments. Values without standard deviations are results from a single trial with 10 recovery tubes per time interval. Z-values were calculated from the plots of the averages of the *D*-values. The *r*-squared values for *z*-value determination were 0.98 or greater.

160F (71.11C) /6 sec
Inactivates:

- 3.4 log
- 9.6 log
- 2.3 log
- 28.5 log
- 2.1 log

- 2.1 log
- 2.3 log

1. Shearer, A.E.H., A.S. Mazzotta, R. Chuyate, & D.E. Gombas. 2002. Heat resistance of juice spoilage microorganisms, J. Food Prot. 8:1271-1275

Heat resistance – different forms of fungal structures

- **Vegetative cells** of yeasts isolated from spoiled acid/acidified products (1)

TABLE 2. *Calculated heat resistance of molds and yeasts in 0.1 M citrate buffer at various pH levels*

Organism	Experimental heating range (°C)	pH	*D*-value (min) at temp 60°C/140°F	*z*-value (°C/°F)
Penicillium citrinum	47.8–55.6	3.0	0.010	4.2/7.5
	47.8–55.6	3.5	0.016	4.6/7.9
	47.8–55.6	4.0	0.009	3.8/6.9
Torulaspora delbrueckii	49.4–56.1	3.0	0.026	4.4/7.9
	50.0–57.2	3.5	0.033	4.4/7.4
	50.0–57.2	4.0	0.018	3.8/6.3
Rhodotorula mucilaginosa	52.8–59.4	3.0	0.120	4.7/8.5
	52.8–59.4	3.5	0.159	4.6/8.2
	50.8–59.4	4.0	0.158	4.5/8.1
Zygosaccharomyces rouxii	ND[a]	3.0	ND[a]	ND[a]
	51.7–58.9	3.5	0.039	3.3/6.0
	53.3–58.9	4.0	0.008	2.1/4.3
Penicillium roquefortii	52.8–61.1	3.0	0.201	4.0/7.2
	52.8–61.1	3.5	0.238	3.7/6.7
	54.9–61.1	4.0	0.290	3.6/6.5
Aspergillus niger	54.4–61.7	3.0	0.451	3.7/6.7
	56.7–61.7	3.5	0.376	3.3/6.0
	54.4–61.7	4.0	0.449	3.6/6.6
Saccharomyces cerevisiae	56.7–63.3	3.0	1.30	3.6/6.6
	58.3–63.6	3.5	2.50	4.0/7.2
	57.8–62.8	4.0	2.80	3.5/6.4

[a] ND, not determined; all values from single final determination following preliminary trials.

160F (71.11C) /6 sec kills

- 2125 log
- 1030
- 141
- 5524
- 206
- 214
- 21

1. Shearer, A.E.H., A.S. Mazzotta, R. Chuyate, & D.E. Gombas. 2002. Heat resistance of juice spoilage microorganisms, J. Food Prot. 8:1271-1275

Heat resistance – different forms of fungal structures

- *Sacchromyces cevesiae* (ascospore) - isolated from heated juice (1)
  - Ascospore: D_{55C} = 106 min; D_{60C} = 6.1 min, z-value = 3.8C° in apple juice
  - Vegetative cells: D_{55C} = 0.90 min
    - ➢ Ascospores were over 100 times heat-resistant than the vegetative cells

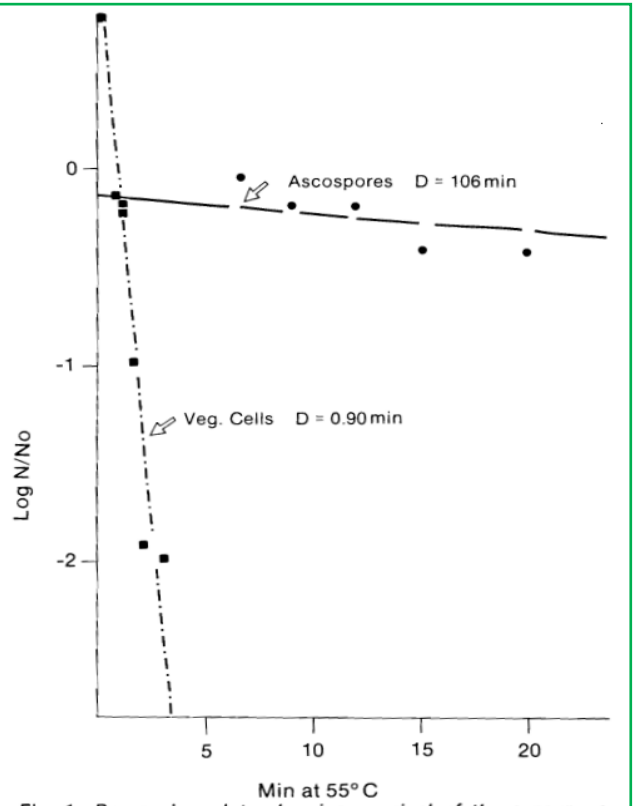


Fig. 1—Regression plots showing survival of the ascospores and vegetative cells of Saccharomyces cerevisiae when heated in an 8.6° Brix apple juice.

Table 1—Heat resistance of ascospores at 60°C in different modifications of apple juice

Heating menstruum	Avg D-value
Control - Distilled water	2.4
Control - 5% aqueous sucrose	4.0
Control - 10% aqueous sucrose	4.6
Apple juice, 8.6° Brix	6.1
9.8° Brix - sucrose added	5.0
13.5° Brix - sucrose added	11.0
18.9° Brix - sucrose added	12.0
8.6° Brix juice plus 6% ethanol	1.2
9% ethanol	0.45
12% ethanol	0.48
8.6° Brix juice - pH 2.4	1.9
pH 3.0	4.6
pH 5.0	4.0
pH 7.2	3.7

160F (71.11C) /6 sec kill:

35 log
21
18
13.7
16 7
7.6
7.0

Assume: z =3.8C for all scenarios

Impact of heating medium composition

1. Splittstoesser , D.F., S.B. Leasor, & K.M.J. Swanson. 1986. Effect of food composition on the heat resistance of yeast ascospores. J. Food Sci. 5:1265-1267

Heat resistance – different forms of fungal structures

- Ethanol impact on yeast ascospore heat resistance:
  - *Saccharomyces* spp. **ascospores** in Beer (4% v/v ethanol) (1):
    - D_{60C} = 6.0 – 11.2 min, z-value = 11.7 – 14.3C

D-value ± SE (min)

Temperature (°C)	*Saccharomyces cerevisiae* DSMZ 1848	*Saccharomyces cerevisiae* DSMZ 70487	*Saccharomyces pastorianus* ATCC 9080	*Saccharomyces cerevisiae* Ethanol Red®
50	62.0 ± 4.51	35.1 ± 1.27	31.3 ± 2.7	34.5 ± 2.97
Adj R^2	*0.920*	*0.983*	*0.883*	*0.870*
MSE	*0.014*	*0.014*	*0.065*	*0.101*
55	28.0 ± 3.14	25.7 ± 2.11	17.3 ± 1.36	19.5 ± 0.43
Adj R^2	*0.785*	*0.898*	*0.994*	*0.900*
MSE	*0.080*	*0.051*	*0.006*	*0.102*
60	11.2 ± 0.57	7.5 ± 0.14	4.6 ± 0.10	6.0 ± 0.54
Adj R^2	*0.961*	*0.993*	*0.896*	*0.993*
MSE	*0.025*	*0.005*	*0.080*	*0.007*
65	3.2 ± 0.55	3.6 ± 0.55	2.2 ± 0.15	2.5 ± 0.07
Adj R^2	*0.577*	*0.706*	*0.946*	*0.941*
MSE	*0.025*	*0.463*	*0.077*	*0.118*
z-value ± SE (°C)	11.7 ± 1.25	14.3 ± 3.01	12.4 ± 1.81	12.7 ± 1.59
Adj R^2	*0.966*	*0.878*	*0.937*	*0.953*
MSE	*0.005*	*0.013*	*0.008*	*0.006*

[a] Values in italic are model performance indices for the parameter estimated. Adjusted R^2 close to 1.0 and low mean square errors (MSE) indicates the goodness of fit.

160F (71.11C) /6 sec Inactivates:	0.08	0.08	0.17	0.12 logs

z-value was much higher than ca. 4.0-6.5C as indicated in the previous slides

Source:
1. Milani et al.2005. Thermal resistance of *Saccharomyces* yeast ascosopores in beers. Int. J. Food Microbiol. 2016:75-80

Heat resistance – different forms of fungal structures

Candida pelliculosa **&** ***Kloeckera apis*** – isolated from fermented pasteurizaed pineapple juice, guava, and passion fruit nectars (1)

Veg. cells or ascospores –not reported

Table 1. Decimal reduction times (D_T) at 55–75°C and z values of *Candida pelliculosa* and *Kloeckera apis* isolated from pasteurized pineapple juice, guava and passion fruit nectars produced in Cameroon

Yeast	Culture media	D_T in minutes at each indicated temperature[a]						z(°C)[b]
		55°C	58°C	60°C	65°C	70°C	75°C	
C. pelliculosa	Pineapple juice (pH 3·95)	nd[c]	nd	4·90±0·01 (r^2=0·98)	3·20±0·01 (r^2=0·999)	3·09±0·01 (r^2=0·99)	1·50±0·01 (r^2=0·99)	31·75±0·03 (r^2=0·91)
	Guava nectar (pH 3·15)	nd	nd	3·62±0·01 (r^2=0·98)	2·49±0·01 (r^2=0·98)	1·87±0·02 (r^2=0·94)	nd	34·84±0·02 (r^2=0·998)
	Passion fruit nectar (pH 3·03)	nd	nd	3·70±0·01 (r^2=0·98)	3·09±0·01 (r^2=0·998)	2·25±0·01 (r^2=0·98)	1·03±0·02 (r^2=0·99)	27·70±0·04 (r^2=0·90)
	Tartaric buffer (pH 2·65)	nd	nd	1·81±0·02 (r^2=0·99)	nd	nd	nd	nd
K. apis	Pineapple juice (pH 3·95)	2·49±0·01 (r^2=0·99)	1·91±0·01 (r^2=0·99)	1·46±0·01 (r^2=0·999)	nd	nd	nd	21·88±0·07 (r^2=0·97)
	Guava nectar (pH 3·15)	2·35±0·01 (r^2=0·997)	1·79±0·01 (r^2=0·999)	1·41±0·02 (r^2=0·99)	nd	nd	nd	22·73±0·04 (r^2=0·99)
	Passion fruit nectar (pH 3·03)	2·38±0·01 (r^2=0·996)	1·89±0·01 (r^2=0·997)	1·60±0·01 (r^2=0·999)	nd	nd	nd	29·07±0·01 (r^2=0·999)
	Tartaric buffer (pH 2·65)	nd	nd	0·47±0·10 (r^2=0·95)	nd	nd	nd	

[a]Each value is the average of two replications.
[b]z Value is the increase in temperature needed to reduce the D_T by 10.
[c]Not determined.

Very high z-values

160F (71.11C) /6 sec Inactivates

- 0.05 log
- 0.06
- 0.07
- 0.22
- 0.22
- 0.15

1. Tchango, J.T., R. Tailliez, P. Eb, T. Njine, & J.P. Homez. 1997. Heat resistance of the spoilage yeasts *Candida pelliculosa* and *Kloeckera apis* and pasteurization values from some tropical fruit juices and nectars. Food Microbiol. 14:93-99.

Heat resistance – different forms of fungal structures

Candida pelliculosa **&** ***Kloeckera apis*** – isolated from fermented pasteurizaed pineapple juice, guava, and passion fruit nectars (1)

Very high z-values obtained - Why?

- A modified "thermal death tube" method":
  - Pre-sterilized glass tube containing 4.5 ml of the heat medium (juice)
  - Inoculated the tube with 0.5 ml of inoculum (10^6-10^8 cfu/ml) from a pre-culture grown at 32C for 48h in juice

    Acid adaptation?
  - Homogenized
  - Preheated to 45C/10min ← Heat adaptation?
  - Heated in a thermostatically-controlled water bath for heat resistance determination
    - So no cell harvesting or washing ← Would "non-washing" help retaining heat resistance?

1. Tchango, J.T., R. Tailliez, P. Eb, T. Njine, & J.P. Homez. 1997. Heat resistance of the spoilage yeasts *Candida pelliculosa* and *Kloeckera apis* and pasteurization values from some tropical fruit juices and nectars. Food Microbiol. 14:93-99.

Heat resistance – different forms of fungal structures

- Mold Conidia – *Aspergillus flavus* & *A. parasiticus* heated in a buffer soln (pH 7.0) (1)
  - D_{60C} = 7.7 – 58.8 sec, z-value = 3.3 – 4.1C°

TABLE 2. *D and z values for conidiospores of* A. flavus *and* A. parasiticus *as determined at various temperatures*

Strain	D value at 45 C (h)	50 C (min)	55 C (min)	60 C (sec)	z value (C)
NRRL 3353	13.97	16.2	3.1	7.7	4.0
NRRL 3161	14.65	34.8	3.8	9.8	4.1
NRRL 3315	52.62	98.2	6.3	19.9	3.8
NRRL 2999	67.28	155.6	8.4	34.8	3.9
NRRL 3251	69.11	188.8	9.5	42.0	4.0
NRRL 482	>161	986.8	28.9	58.8	3.3

160F (71.11C) /6 sec
Inactivates:

- 166 logs
- 100 logs
- 252 logs

1. Doyle , M.P.& E.H. Marth, 1975. Thermal Inactivation of Conidia from *Aspergillus flavus* and *Aspergillus parasiticus*. J. Milk Food Technol. 11:678-682

Heat resistance – different forms of fungal structures

- Mold Conidia – *Aspergillus flavus* & *A. parasiticus* heated in a buffer soln (pH 7.0) (1)

TABLE 3. *D values determined at 55 C for 7-, 10-, 15-, and 20-day-old conidiospores produced on Moyer's medium by selected strains of* A. flavus *and* A. parasiticus

	D Value (min) for conidiospores of different ages			
Strain	7 days	10 days	15 days	20 days
NRRL 3353	2.8	3.1	2.8	2.4
NRRL 3315	4.1	6.3	2.5	1.5
NRRL 2999	9.0	8.4	7.2	4.1

TABLE 4. *D values at 55 C for 10-day-old condiospores produced on various media by* A. flavus *and* A. parasiticus

	D values (min) of conidia produced on				
Strain	Mycological	Czapek's	Moyer's	Y-M	PDA
NRRL 3353	1.0	2.6	3.1	1.8	2.9
NRRL 3315	1.3	5.1	6.3	1.4	2.3
NRRL 2999	4.1	4.8	8.4	3.3	5.6

As conidia got older, the heat resistance decreased

"Moyer's medium is a very nutritious substrate"

- Conidia produced on "a medium with a substantial amount of sugar were more heat resistant than those formed when the amounts of sugar was less"
- "Conidia produced on media that contained a relatively large amount of protein and amino acids were less heat resistant than those produced without any or with much smaller amounts od exogenous protein present in the medium"

1. Doyle , M.P.& E.H. Marth, 1975. Thermal Inactivation of Conidia from *Aspergillus flavus* and *Aspergillus parasiticus*. I. Effects of moist heat, age of conidia, and sporulation medium. J. Milk Food Technol. 11:678-682 .

Heat resistance – different forms of fungal structures

- Mold Conidia – *Aspergillus flavus* & *A. parasiticus* heated in a buffer soln (pH 7.0) (1)

TABLE 4. *D-values of 55 C for 10-day old conidiospores of* A. flavus *and* A. parasiticus *produced on Moyer's medium and heated at different pH values achieved by several buffers*

Buffer	pH	D value (min) for strains NRRL 3353	NRRL 3315	NRRL 2999
KH_2PO_4 and NaOH	7.0	3.1	6.4	8.4
Na acetate and acetic acid	3.5	0.9	1.3	5.8
	4.5	2.4	6.1	6.5
	5.5	2.6	6.0	7.8
Citric acid and $Na_2HPO_4 \cdot 2H_2O$	3.5	3.0	6.7	17.7
	4.5	3.7	4.2	14.4
	5.5	2.9	3.7	10.6
	6.0	—[a]	—	7.5
KHP-HCl and KHP-NaOH	3.5	3.3	3.9	8.6
	4.5	3.5	3.9	5.9
	5.5	1.9	3.6	5.2

[a]-indicates not tested.

Impact of pH, Sucrose, & NaCl levels

TABLE 6. *D-values at 55 C for 10-day old conidiospores of* A. flavus *and* A. parasiticus *produced on Moyer's medium and heated when various amounts of sucrose were in the heating menstruum*

Concentration (w/w, %)	D value (min) for strains NRRL 3353	NRRL 3315	NRRL 2999
0.0	3.1	6.3	8.4
10.0	3.7	6.4	12.2
30.0	4.6	10.0	25.2
45.0	14.5	28.7	63.4
60.0	65.7	84.2	199.0

TABLE 7. *D-values of 55 C for 10-day old conidiospores of* A. flavus *and* A. parasiticus *produced on Moyer's medium and heated when various amounts of glucose were in the heating menstruum*

Concentration (w/w, %)	D value (min) for strains NRRL 3353	NRRL 3315	NRRL 2999
0.0	3.1	6.3	8.4
10.0	5.1	7.0	14.5
30.0	9.9	24.2	39.2
45.0	24.4	57.6	95.9
60.0	66.2	117.9	213.9

1. Doyle , M.P.& E.H. Marth, 1975. Thermal Inactivation of Conidia from *Aspergillus flavus* and *Aspergillus parasiticus*. II. Effects of pH and buffers, glucose, sucrose, and sodium chloride. J. Milk Food Technol. 12:750-758 .

Heat resistance – different forms of fungal structures

- Mold Conidia – *Penicillium citrinum*, *P. roqueforti*, *Aspergillus niger*
  - Heated in citrate buffer (pH 3.0, 3.5, 4.0) (1)
  - z-value not reported

TABLE 2. *Calculated heat resistance of molds and yeasts in 0.1 M citrate buffer at various pH levels*

Organism	Experimental heating range (°C)	pH	*D*-value (min) at temp 60°C/140°F	*z*-value (°C/°F)
Penicillium citrinum	47.8–55.6	3.0	0.010	4.2/7.5
	47.8–55.6	3.5	0.016	4.6/7.9
	47.8–55.6	4.0	0.009	3.8/6.9
Torulaspora delbrueckii Veg cells	49.4–56.1	3.0	0.026	4.4/7.9
	50.0–57.2	3.5	0.033	4.4/7.4
	50.0–57.2	4.0	0.018	3.8/6.3
Rhodotorula mucilaginosa	52.8–59.4	3.0	0.120	4.7/8.5
	52.8–59.4	3.5	0.159	4.6/8.2
	50.8–59.4	4.0	0.158	4.5/8.1
Zygosaccharomyces rouxii Veg cells	ND[a]	3.0	ND[a]	ND[a]
	51.7–58.9	3.5	0.039	3.3/6.0
	53.3–58.9	4.0	0.008	2.1/4.3
Penicillium roquefortii	52.8–61.1	3.0	0.201	4.0/7.2
	52.8–61.1	3.5	0.238	3.7/6.7
	54.9–61.1	4.0	0.290	3.6/6.5
Aspergillus niger	54.4–61.7	3.0	0.451	3.7/6.7
	56.7–61.7	3.5	0.376	3.3/6.0
	54.4–61.7	4.0	0.449	3.6/6.6
Saccharomyces cerevisiae Veg cells	56.7–63.3	3.0	1.30	3.6/6.6
	58.3–63.6	3.5	2.50	4.0/7.2
	57.8–62.8	4.0	2.80	3.5/6.4

[a] ND, not determined; all values from single final determination following preliminary trials.

160F (71.11C) /6 sec kills

- 2125 log
- 1030
- 141
- 5524
- 206
- 214
- 21

1. Shearer, A.E.H., A.S. Mazzotta, R. Chuyate, & D.E. Gombas. 2002. Heat resistance of juice spoilage microorganisms, J. Food Prot. 8:1271-1275

Heat resistance – different forms of fungal structures

- Mold Conidia – *Penicillium expansum* heated in a apple juice (pH 3.25, 12.2 brix) (1)
  - z = 7.57C

T (°C)	*D* (min)
50	10.68
52	6.64
54	3.32
56	1.14
60	0.61

- z = 7.57C

160F (71.11C) /6sec Inactivates:

- 5.5 log

1. Salomao, B.C.M., J.J. Churey, G.M.F. Aragao, and R. Worobo. 2009. Modeling *Penicillium expansum* resistance to thermal and chlorine treatments. J. Food Prot. 12:2618-2622

Heat resistance – different forms of fungal structures

- Mold Conidia – *Botrytis cinerea* heated in strawberry puree (pH 3.87, Brix 9, aw 0.985) (1)

Table 2
Calculated *D* values of the first order kinetics equation for the thermal inactivation of *B. cinerea* conidia in a sterile strawberry puree (SSP), and in a synthetic medium (SM).

Medium	First order kinetics			
	T (°C)	*D* (min)*	R^2	z-value
Sterile strawberry puree (SSP)	42	44.9 ± 5.8	0.98	
	44	13.8 ± 3.6	0.99	
	46	4.7 ± 0.7	0.99	4.15C
	48	1.7 ± 0.5	0.91	
Synthetic medium (SM)	42	22 ± 5.8	0.99	
	44	8.5 ± 2.3	0.95	
	46	4.1 ± 0.2	0.98	5.08C
	48	1.4 ± 0.1	0.97	

* Means ± standard deviation from three replicates.

160F (71.11C) /6sec
Inactivates:

1197 logs

1. Villa-Rojas, R., M.E. Sosa-Morales, A. Lopez-Malo, & J. Tang. 2012. Thermal inactivation of *Botrytis cinerea* conidia in synthetic medium and strawberry puree_2012, Int. J. Food Microbiol. 155:269-272

Heat resistance – different forms of fungal structures

Heat resistance comparison – different fungal forms of existence

- Mold Conidia – *Botrytis cinerea* and *Monilinia fructigena* heated in a phosphate buffer (pH

Table 1
The *D* values for *B. cinerea* at different temperatures

Temperature (°C)	*D* value (min)
40	29.959
43	6.782
45	2.559
48	0.607

z-value

4.65C

Table 2
The *D* values for *M. fructigena* at different temperatures

Temperature (°C)	*D* value (min)
39	21.697
41	7.302
43	2.492
45	0.862

z-value

4.17C

140F (60C) /6sec
Inactivates:

- 66 logs
- 500 logs

1. Marquenie, D., J. Lammertyn, A.H. Geeraerd, C. Soontjens, J.F. Van Impe, B.M. Nicolai, & C.W. Michiels. 2002. Inactivation of conidia of *Botrytis cinerea* and *Monilinia fructigena* using UV-C and heat treatment. Int. J. Food Microbiol. 74:27-35

Heat resistance – different forms of fungal structures

Heat resistance comparison – different fungal forms of existence

- Mold Conidia - *Penicillium puberulum, Aspergillus flavus*, and *Geotrichum candidum*
  - Heated in a phosphate-Tween buffer (1)

TABLE 1. *Effects of preservatives on D-values of molds heated in media adjusted to pH 2.5 to 4.5.*

Organism	Preservative	Concentration (ppm)	Decimal reduction times (min) at various pH values[a] 2.5	3.0	3.5	4.0	4.5
P. puberulum	Control	0	28.7 efgh	29.6 cdef	29.5 cdef	30.8 bcde	32.8 a
	Potassium sorbate	50	29.3 cdefg	29.0 defgh	29.4 cdefg	30.6 cde	32.6 ab
		100	22.6 j	24.9 i	28.8 defgh	30.1 cdef	31.4 abc
	Sodium benzoate	50	20.7 k	23.8 ij	28.6 fgh	30.4 cdef	30.9 abcd
		100	9.0 n	12.8 m	17.7 l	27.5 gh	27.4 h
A. flavus	Control	0	40.8 bc	41.7 abc	43.2 ab	44.4 a	44.7 a
	Potassium sorbate	50	20.8 h	27.5 f	36.6 d	40.8 bc	43.2 ab
		100	18.9 hi	25.9 fg	30.9 de	39.9 c	42.6 abc
	Sodium benzoate	50	15.2 jk	24.1 g	33.2 e	34.2 de	34.9 de
		100	8.1 l	9.5 l	14.4 k	17.6 ij	20.9 h
G. candidum	Control	0	6.7 gf	23.0 b	28.7 a	30.8 a	31.2 a
	Potassium sorbate	50	6.2 gf	6.7 gf	12.5 e	15.8 d	19.2 c
		100	5.2 g	5.5 g	9.0 f	13.8 de	14.0 de

[a]Considering each organism separately, D-values not followed by the same letter are significantly different ($P \leq 0.05$).

Temp. at which D-values were obtained

49C

52C

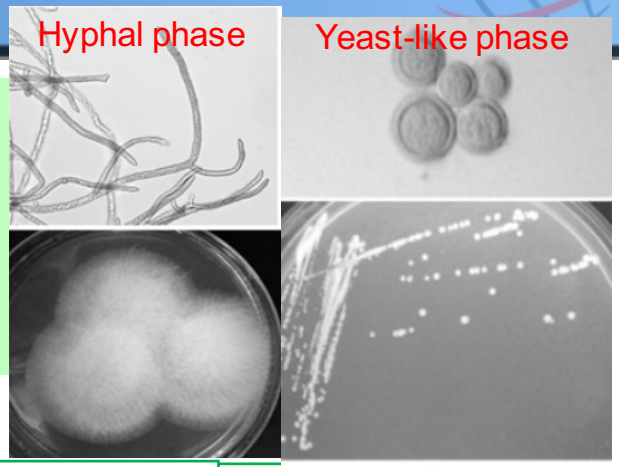
52C

No z-value reported

1. Beuchat , L.R..1981. Influence of potassium sorbate and sodium benzoate on heat inactivation of *Aspergillus flavus*, *Penicillium puberulum* and *Geotrichum candidum*. J. Food Prot.44:450-454.

Heat resistance – different forms of fungal structures

- *Mucor circinelloides* (dimorphoric) - heated in skim milk (1)
  - Mold hyphae/sporangiospores
  - Yeast-like cells



The *D*- and *z*-values for hyphal phase and yeast-like phase *Mucor circinelloides* yogurt spoilage isolate in skim milk.

	D-value (min)	*z*-value (°C)
Hyphal phase		
58 °C	0.94 ± 0.53	3.09
56 °C	10.17 ± 0.28	
54 °C	38.31 ± 0.02	
Yeast-like phase		
55 °C	2.44 ± 0.35	0.34
53 °C	6.87 ± 1.19	
51 °C	14.25 ± 0.12	

160F (71.11C) /6 sec Kills

- 900 log

The mold can exist as resting asexual chlamydospores and arthrospores, as well as sexual zygospores

1. Snyder, A.B., J.J. Churey, & R.W. Worobo. 2016. Characterization and control of *Mucor circinelloides* spoilage in yogurt. Int. J. Food Microbiol. 228:14-21

Heat resistance – different forms of fungal structures

- Chlamydospores / Scalerotia: Asexual, resting spores/cells
  - No actual heat resistance values reported
  - Expert view: more heat resistant than vegetative cells or conidia
  - *Mucor* / *Amylomyces* / *Paecilomyces*, etc. can form many chlamydospores

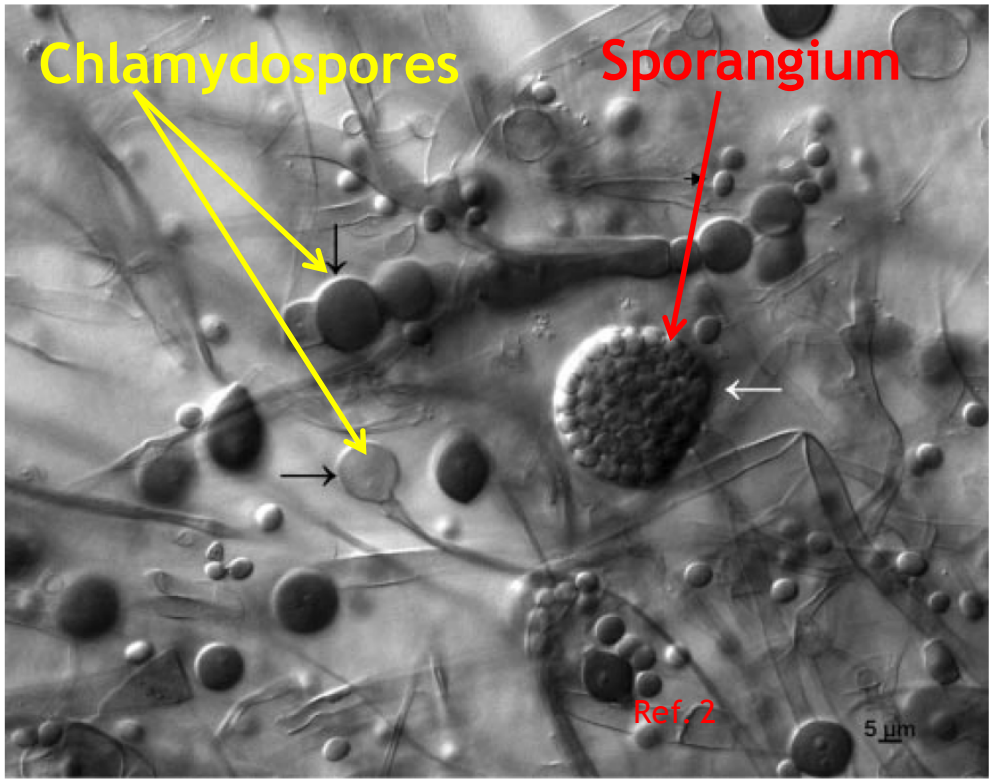


FIG. 2. Microscopic morphology of *Mucor circinelloides* on PDA after 6 days at 30°C, showing sporangium (white arrow), sporangiospores (black arrowhead), and chlamydospores produced singly and in short chains (black arrows). Nomarski optics were used. Bar, 5 µm.

1. Hesseltine, C.w. and C. K. Featherston. 1985. Anaerobic growth of molds isolated from fermentation starters used for foods in Asian countries. Mycologia 77:390-4000
2. Iwen, P.C., LL. Sigler, R.K. Noel, and A.G. Freifeld. 2007. *Mucor circinelloides* was identified by molecular methods as a cause of primary cutaneous Zygomycosis. J. Clin. Microbiol. 45:636-640
3. Ruyle, E.H., W.E. Pearce & G. L. Hays. 1946. Prevention of mold in kettled blueberries in no. 10 cans. J. Food Sci. 11:274-279
4. Y. Kikoku, N. Tagashira, & H. Nakano. 2008. Heat resistance of fungi Isolated from frozen blueberries. 71:2030-2035.

Heat resistance – different forms of fungal structures

- 1938 Canned (#10) Blueberry Spoilage Isolates:
  - Formed sclerotia (called "**Sclerotia**" strain) (3)

Heat Resistance of Ascospores and Sclerotia

Temperature	74°C. (165°F.)		78°C. (171°F.)		81°C. (177.8°F.)		85°C. (185°F.)		90.5°C. (195°F.)		93.3°C. (200°F.)		100°C. (212°F.)	
	+	—[1]	+	—	+	—	+	—	+	—	+	—	+	—
Ascospores, 160,000 per ml.....	210′	240′	65′	70′	10′	15′	....	....	....	....	....	....	....	....
Sclerotia, 86,000 per ml.....	....	....	....	....	....	....	270′	300′	30′	40′	9′	10′	0	1′

z = 10.6F

z = 10.3F

[1] The + sign means growth and the — sign means no growth when heated for the number of minutes shown at the temperature indicated.

1. Ruyle, E.H., W.E. Pearce & G. L. Hays. 1946. Prevention of mold in kettled blueberries in no. 10 cans. J. Food Sci. 11:274-279
2. Kikoku Y., N. Tagashira, & H. Nakano. 2008. Heat resistance of fungi Isolated from frozen blueberries. 71:2030-2035.
3. Williams, C.C., E.J. Cameron, & O.B. Williams. 1941. A FACULTATIVELY ANAEROBIC MOLD OF IJNUSUAL HEAT RESISTANCE. J. Food Sci. 6:69-73

Heat resistance – different forms of fungal structures

- 1938 Canned (#10) Blueberry Spoilage Isolates:
  - Formed sclerotia (called "Sclerotia" strain) (3)

- The canned blueberry spoilage mold isolates grew:
  - Only on surface inside the can
  - Only in enamel-lined cans
    - Not in plain cans - headspace O2 was consumed via reacting with can metal (3)
  - Can grow under 0.5% O_2 (1)
- Two strains (*Penicillium*) were isolated (3)
  - One produced ascospores
  - Another produced sclerotia
    - Sclerotia strain - much more heat resistant
- "The mold sclerotia may survive a temperature of 85C (185F) for a few hours and 87.8C (190F) for about one hour" (1)

1. Ruyle, E.H., W.E. Pearce & G. L. Hays. 1946. Prevention of mold in kettled blueberries in no. 10 cans. J. Food Sci. 11:274-279
2. Kikoku Y., N. Tagashira, & H. Nakano. 2008. Heat resistance of fungi Isolated from frozen blueberries. 71:2030-2035.
3. Williams, C.C., E.J. Cameron, & O.B. Williams. 1941. A FACULTATIVELY ANAEROBIC MOLD OF IJNUSUAL HEAT RESISTANCE. J. Food Sci. 6:69-73

Heat resistance – different forms of fungal structures

- 1938 Canned (#10) Blueberry Spoilage Isolates:

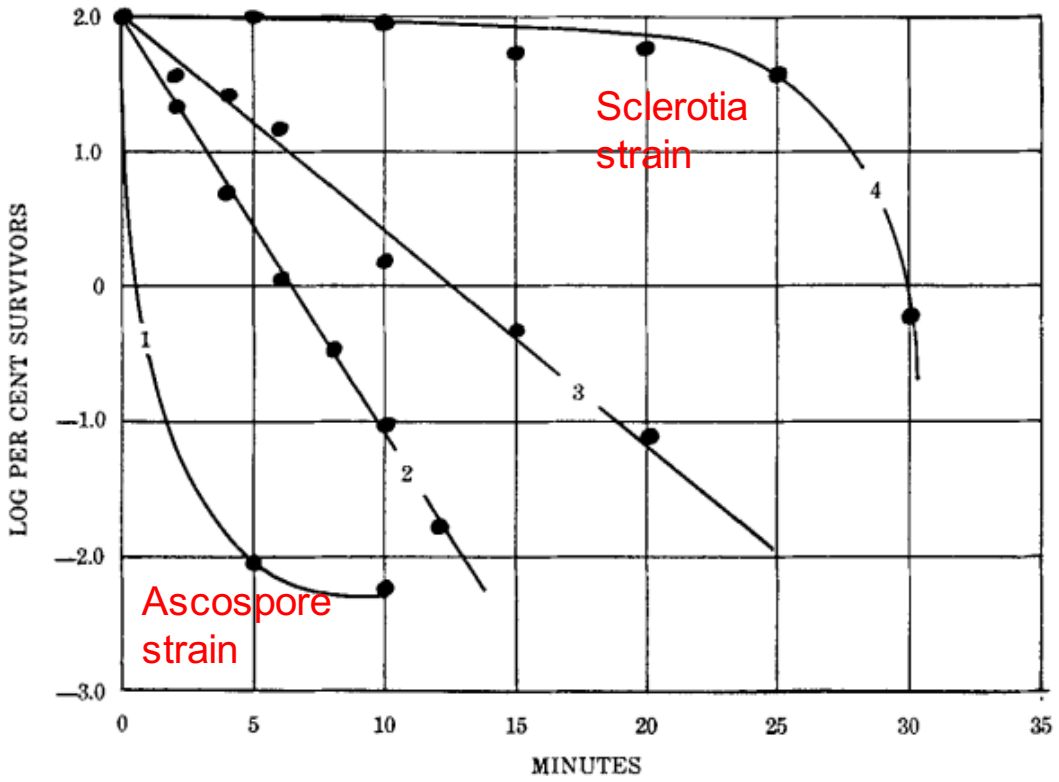


FIG. 2. Typical destruction rate curves for various organisms.
1—Ascospores at 81°C.(177.8°F.)
2—Putrefactive Anaerobe No. 3679 at 115°C.(239°F.)
3—*Escherichia coli* at 51.7°C.(125°F.)
4—Sclerotia at 90.5°C.(195°F.).

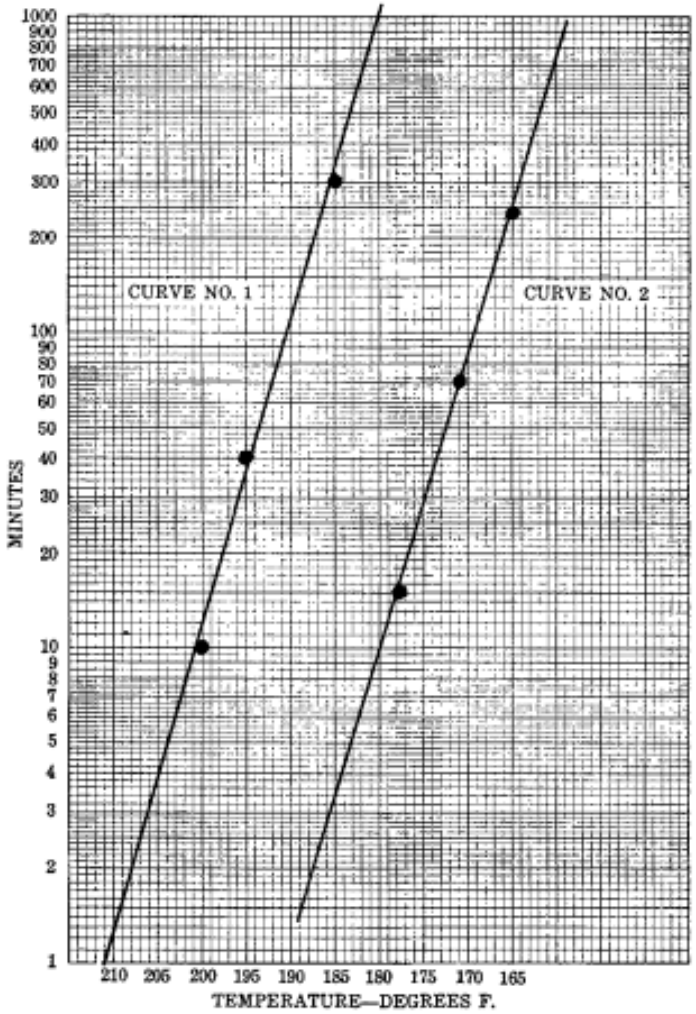
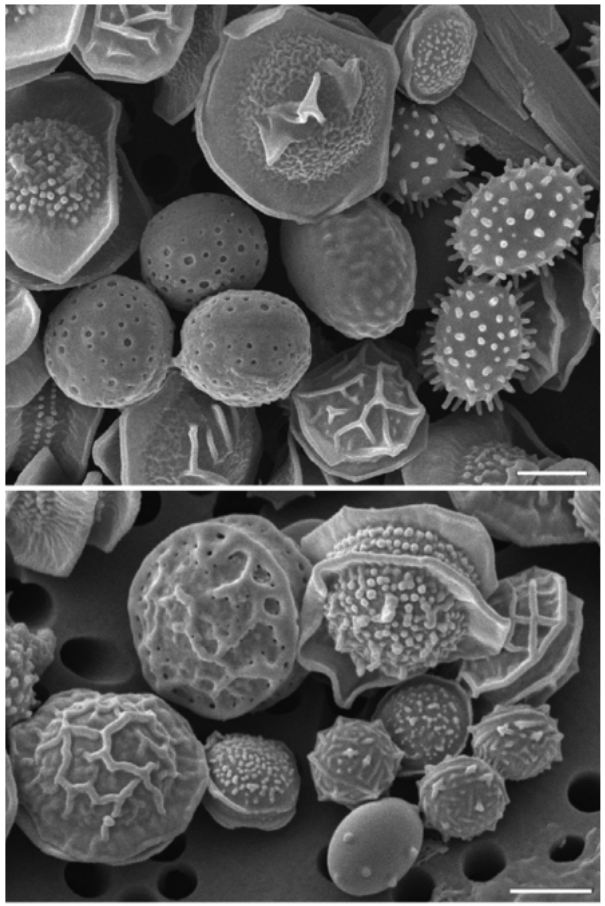


FIG. 1. Thermal death-time curves.
Sclerotia—Curve No. 1 z = 10.3 E = 1,000
Ascospores—Curve No. 2 z = 10.6 E = 9.7.

1. Ruyle, E.H., W.E. Pearce & G. L. Hays. 1946. Prevention of mold in kettled blueberries in no. 10 cans. J. Food Sci. 11:274-279
2. Williams, C.C., E.J. Cameron, & O.B. Williams. 1941. A FACULTATIVELY ANAEROBIC MOLD OF IJNUSUAL HEAT RESISTANCE. J. Food Sci. 6:69-73

Heat resistance – different forms of fungal structures: - Mold Ascospores

- Mold Ascospores: Sexual spores
  - Very high heat resistance: D_{90C} ranges from 1 to 12 min, with z- value 6-7C (1)
  - Caused a lot of spoilages in thermally processed juices or beverages



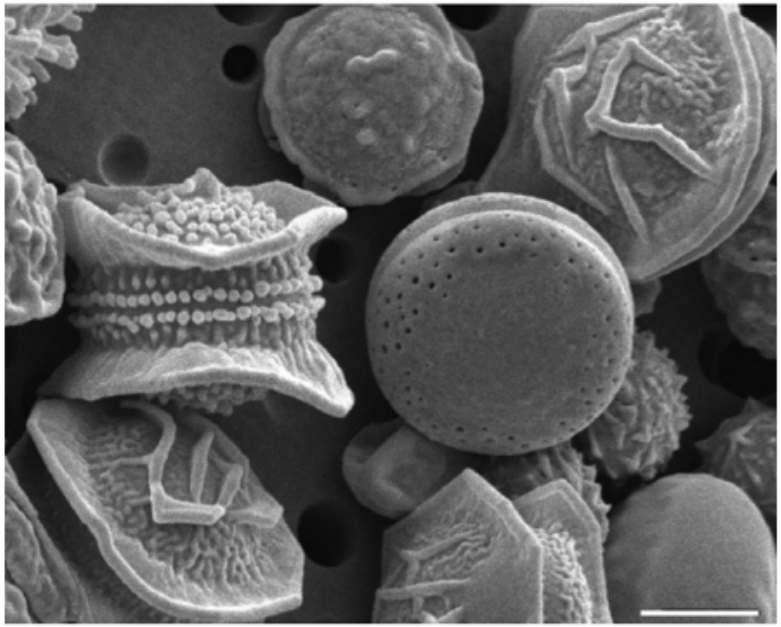


Figure 2.7 cryoSEM micrographs illustrating the variability in morphology in a mixture of ascospores of 25 fungal species belonging to the genera *Neosartorya*, *Eurotium*, *Talaromyces*, and *Thermoascus*. Bars are 2 μm.

1. Pitt, J.I. & A.D. Hocking. 2009. Chapter 2. The Ecology of Fungal Food Spoilage (page 3-9). In "Fungi and Food Spoilage," by J.I. Pitt & A.D. Hocking. 3rd ed. Springer Science.
2. Source of graph - Wyatt, T.T., H.A.B. Wosten, J. Dijksterhuis. Fungal spores for dispersion in space and time. Adv. Appl. Microbiol. 85:43-91.

Heat resistance – different forms of fungal structures:
- Mold Ascospores

Ascospores inside Asci, which are inside Ascomata

- Ascomata: Cleistothecia or Gymnothecia

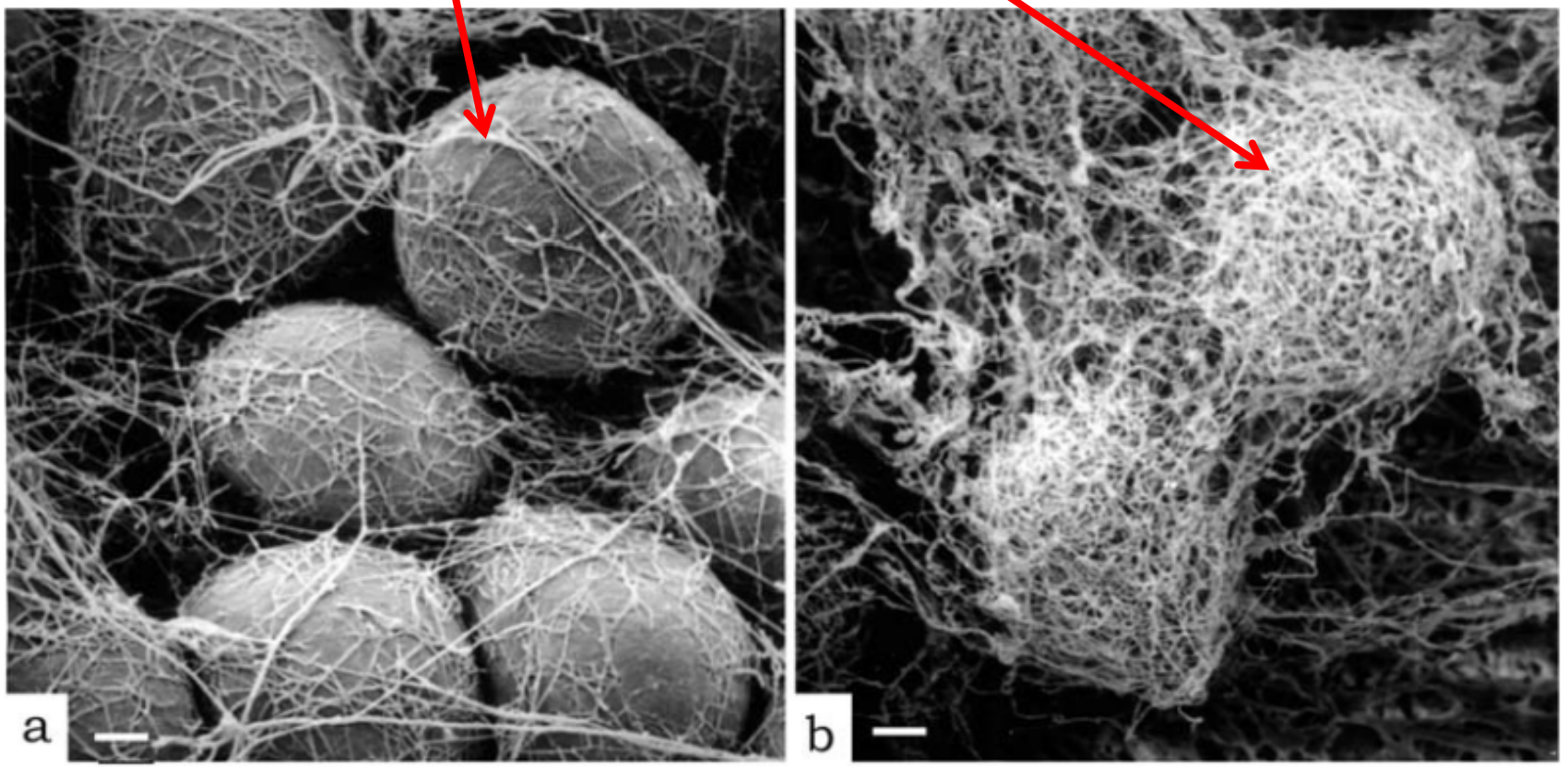


Fig. 3.3 **(a)** Cleistothecia of *Eupenicillium*; **(b)** gymnothecia of *Talaromyces*. SEM. Bars = 50 µm

Heat resistance – different forms of fungal structures:
- Mold Ascospores

Ascospores heat resistance

- **A lot of studies** (see the listed references)

Table 2 Heat resistance of *Byssochlamys fulva, Byssochlamys. nivea,* and *Byssochlamys. spectabilis* ascospores (ref. 5)

Species	*Heating medium*	*Heat resistance*
Byssochlamys fulva	Glucose (16° Brix), tartaric acid (33 mM), pH 3.6 and 5.0	90 °C, 1.2–46 min 3 $\log_{10}$ inactivation time
	Tomato juice	90 °C, 8.1 min 1 $\log_{10}$ inactivation time
	Grape juice	$D_{87.8\,°C}$, 11.3 min
Byssochlamys nivea	Grape juice	88 °C, survived 60 min
	Apple juice	99 °C, survived in juice containing 4.7% sucrose
	Cream (10% w/w fat)	$D_{92\,°C}$, 1.6–19 s
	Tomato juice	90 °C, 1.5 min 1 $\log_{10}$ inactivation time
Byssochlamys spectabilis	ACES[a] buffer (10 mM), pH 6.8	$D_{85\,°C}$, 47–75 min

[a]ACES: *N*-[2-acetamido]-2-aminoethane-sulfonic acid.

1. Frac, M., S. Jezierska-Tys, & T. Yaguchi. 2015. Occurrence, detection, and molecular and metabolic characterization of heat resistant fungi in soils and plants and their risk to human health. Adv. In Agronomy. 132:161-204
2. Dijksterhuis, J. 2007. Chapter 5 - Heat-resistant ascospore, in "Food Mycology – A Multifaceted Approach to Fungi and Food," J. Dijksterhuis & R. A. Samson (editors). CRC Press. pp.101-117
3. Dijksterhuis, J. 2017. The fungal spore and food spoilage. Current Opinion in Food Sci. 17:68-74
4. Tournas, V. 1994. Heat-Resistant Fungi of Importance to the Food and Beverage Industry. Crit. Rev. Microbiol. 20:243-263
5. Kotzkidou. 2014. *Byssochlamys*. Encyclopedia of Food Microbiology, 3nd ed. pp. 344 – 350
6. Wyatt, T.T., H.A.B. Wosten, J. Dijksterhuis. Fungal spores for dispersion in space and time. Adv. Appl. Microbiol. 85:43-91.

Heat resistant molds (HRM) important to beverage spoilage

Teleomorph	Anamorph
Byssochlamys fulva	*Paecilomyces fulvus*
Byssochlamys nivea	*Paecilomyces niveus*
Byssochlamys spectabilis	*Paecilomyce variotii*
Neosartorya fischeri var. *fischeri*	*Aspergillus fischerianus*
Neosartorya fischeri var. *globra*	*Aspergillus fischeri* var. *glaber*
Neosartorya fischeri var. *spinosa*	
Neosartorya fumigata	*Aspergillus fumigatus*
Petromyces parasiticus	*Aspergillus parasiticus*
Emericella nidulans	*Aspergillus nidulans*
Petromyces flavus	*Aspergillus flavus*
Talaromyces flavus	*Penicillum dangeardii*
Talaromyces macrosporus	*Penicillum macrosporum*
Talaromyces bacillisporus	*Geosmithia swiftii*
Talaromyces trachyspermus	*Penicillum lehmannii*

Teleomorph	Anamorph
Eupenicillium brefeldianum	*Penicillium dodgei*
Eupenicillium cinnamonpurpureum	*Penicillium phoeniceum*
Eupenicillium hirayamae	*Penicillium hirayamae*
Eupenicillium javanicum	*Penicillium indonesiae*
Eupenicillium orchrosalmoneum	*Penicillium orchrosalmoneum*
Eurotium	*Aspergillus*
Eurotium herbariorum	*Aspergillus*
Eurotium rubrum	*Aspergillus rubrobrunneus*
Eurotium chevalieri	*Aspergillus chevalieri*
Emericella nidulans	*Aspergillus nidulans*
Monascus	*Basipetospora*
Monascus ruber	*Basipetospora rubra*
Thermoascus	*Paecilomyces*
Thermoascus crustaceus	
Hamigera striatus	
Chaetomium	*Botryotrichum*

Heat resistance - Ascospores

- Requires high heat to inactivate HRM in products
- Controlling HRM by heat may negatively impact product sensory?

Tabel 1. Heat-resistance of ascospores at different temperatures and medium composition.

Fungal species	T	D-value	Medium	Reference
Byssochlamys fulva	86°	13-14	Grape Juice	1
	90°	4-36*	Buffer pH 3.6 and 5.0, 16°Brix	2
		8	Tomato juice	3
B. nivea	85°	1,3-4,5	Buffer pH 3.5	4
		34,6	15º Brix Strawberry pulp	5
	88°	8-9 sec	Ringer solution	6
	90°	1,5	Tomato juice	3
B. spectabilis	85°	ca. 70	Buffer, pH 6,8	7
Eurotium herbariorum	70°	1,1 – 4,6	Grape Juice, 65°Brix	8
Eupenicillium javanicum	85°	3,7	15º Brix Strawberry pulp	5
Monascus ruber	80°	1,7 – 2,0	Buffers (pH 3,0 ; pH 7,0)	9
		0,9 – 1,0	In brine	
Neosartorya fischeri	85°	13,2	Apple Juice	10
		10,1	Grape Juice	10
		10-60	In ACES-buffer, 10 mM, pH 6.8	11
		10,4	Buffer pH 7.0	10
		14,5	15º Brix Strawberry pulp	5
		15,1	15º Brix Apple Juice	12
		19,6 – 29,5	Dionized water, pineapple juice and concentrate	13
		35,3	Buffer pH 7.0	14
	88°	1,4	Apple Juice	15
		4,2-16,2	Heated fruit fillings	16
		12,4 – 17,0	Dionized water, pineapple juice and concentrate	13
	90°	4,4-6,6	Tomato Juice	3
	91°	<2	Heated fruit fillings	16
N. pseudofischeri	95°	20 sec		7
Talaromyces flavus (macrosporus)	85°	3,3	15º Brix Strawberry pulp	5
	85°	39	Buffer pH 5.0, glucose, 16º	17
		20-26	Buffer pH 5.0, glucose	18
	88°	7,8	Apple Juice	15
		7,1 – 22,3	Heated fruit fillings	16
	90°	2-8	Buffer pH 5.0, glucose	18
		6,2	Buffer pH 5.0, glucose	10
		6,0	Buffer pH 5.0, glucose. Slug flow heat exchanger	10
		2,7 – 4,1	Organic acids	19
		2,5 – 11,1	Sugar content (0-60º Brix)	19
		5,2 – 7,1	PH 3.6-6.6	19
	91°	2,1 – 11,7	Heated fruit fillings	16
T. helicus	70°	ca. 20		20
T. macrosporus	85°	30-100	In ACES-buffer, 10 mM, pH 6.8	21
T. stipitatus	72°	ca. 85		20
T. trachyspermus	85°	45 sec		17
Xeromyces bisporus	82.2°	2,3		22

1. Dijksterhuis, J. 2007. Heat-resistant ascospore. In "Food Mycology – A Multifaceted Approach to Fungi and Food," J. Dijksterhuis & R. A. Samson (editors). CRC Press. pp.101-117

Fungal dispersal & airborne mold

(A way to understand airborne fungi)

Fungal dispersal & airborne mold

Understand sources of fungi based on their dispersal

- **Air dispersal** – e.g., *Aspergillus*, *Penicillium*, *Cladosporium*
  1. Exposing dry spores masses to air current
  2. Shooting spores into air via water pressure
  3. Carried by tiny water droplets in the air (foggy)

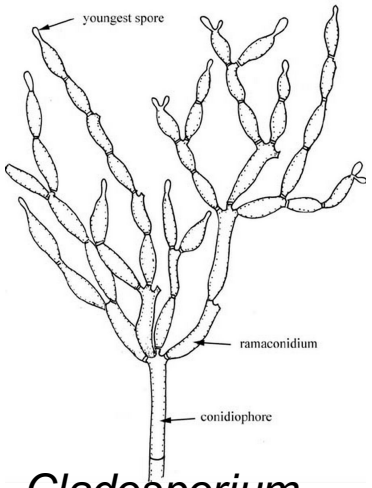
- *Aspergillus*, *Penicillium*, *Cladosporium* - dominating fungal species in plant/indoor/outdoor air
- *Aspergillus* produces large quantities of conidia: >10,000 conidia/conidiophore
- Outdoor air: Over 10 *Aspergillus fumigatus* spores/m^3

- **Water dispersal:** *Fusarium*, *Verticillium*
- **Insect dispersal**

- **Decaying plants**
  - Tremendous mold activity in the fall due to death of many plants

Spore-forming structures (conidiophores) produced by *Penicillium van oranjei* as visualized by cryo-SEM. Long stipes lift specialized cells into the air that produce rows of airborne conidia. Through multiplication of growing apices, more rows can be formed simultaneously.

Dijksterhuis, J. 2017. The fungal spore and food spoilage. Curr. Opinion in Food Sci. 17:68-74



Cladosporium

Fungal survival /stress resistance mechanisms

Fungal survival /stress resistance mechanisms

- **Accumulation of compatible solutes, such as polyols, e.g., mannitol and glycerol, trehalose, etc.**
  - Compatible solutes can reduce cytoplasmic Aw and stabilize cytoplasmic structures
  - Decreased cytoplasmic Aw may result in increased heat resistance
  - *Neosartorya fischeri* ascospores - trehalose-based oligosaccharide (a novel compatible solute)

- **Produce stress adaptation proteins,** such as heat shock proteins

- **Dormant /resting state: Conidia, Chlamydospores, Sclerotia, Ascospores**
  - Low or absence of metabolic activities; Lower intracellular aw
  - Thick cell walls
  - Pigmented cell wall
  - These dormant structures may still change over time
  - Extremely stress-resistance ascospores - *Talaromyces macrosporus* survived 17 yrs in dark at RT
  - If fungi meet periods of very low nutrients or conditions that surpass the limits of growth, they produce stabilized cells for survival without growth
    - Stabilization is characterized by: lowering of metabolic activity, absence of cellular extension, increase of stress resistance
    - Stabilized cells – in a state of dormancy

Fungal survival /stress resistance mechanisms

- **Ways to break "dormant" fungal ascospores (i.e., "activation")**
  - **Heat shock** - the temp, duration, and heat shock media can be different for different species
    - e.g., 75-80° C for 10-30 min
  - Exposure to chemicals, e.g., sanitizers
  - High pressure treatment
  - Varies among different fungal species

Beverage pasteurization = heat shock HRM

1. Wyatt, T.T., H.A.B. Wosten, J. Dijksterhuis. Fungal spores for dispersion in space and time. Adv. Appl. Microbiol. 85:43-91.

Factors affecting heat resistance

A question to consider:

- **Will the heat resistance data obtained in lab really reflect what the fungi are in their natural states?**

Factors affecting fungal heat resistance

- **History of spore formation accounts:** Conditions of spore formation affect properties of spores
  - Growth media - composition of growth media, Brix/high sugar, pH, Aw
  - Growth temperature
    - Conidia of *A. fumigatus* grown at 25, 27, and 45C, show clear difference in resistance to UV / H_2O_2 /heat, trehalose accumulation, formation of mycotoxin
- **Age:**
  - Heat resistance of conidia or ascospores may increase with age

Factors affecting fungal heat resistance

- **How to prepare spore crops in lab may give different heat resistance data**
  - **Lab-cultured spores** may not have the same physiological state as **those naturally existed in environment**
    - Sporulation and post-sporulation conditions
    - Rehydration
    - Exposure to light
    - Fluctuating moisture and temp
    - Interaction between fungi and environment or between fungi and other organisms
  - **Natural state: Ascomata, asci, ascospores**
    - Many heat resistance studies break open (e.g., with French press) ascomata/asci to obtain free ascospore suspension

A question to ask yourself: Does a heat resistance study really provide the data that you can use to design a process? How to prepare the culture / design the test - critical

Factors affecting fungal heat resistance

- **Natural dry state vs rehydrated state** (e.g., in heat resistance studies)
  - **Harvesting *P. digitatum* conidia** (grown on PDA agar) by dry collection (on lid) (i.e., non-hydrated conidia) vs water flush conidia off agar (i.e., rehydrated conidia):
    - The former survives 0.30 kPa ethanol vapor pressure for 24 and 48h, while the latter showed 3.8 log decrease.
    - Possible loss of compatible solutes from fungal spores when exposed to an environment of a water potential higher than that inside their cells.

  Non-hydrated scenario: Bottle air-rinsed before hot fill

  - Conidia of *Aspergillus fumigatus*:
    - Dry harvesting from dried-out agar showed nearly unchanged germination behavior when stored at ambient temp
    - When stored in water, the conidia showed 25% less outgrowth after ten days of storage
  - Ascospores or conidia in nature may have sufficient periods of aging
    - Nature/soil may harbor the most heat resistant ascospores
  - Laboratory growing & harvesting conditions, such as aw (for xerophiles), washing (isotonic or not), etc., can affect fungal crop physiological states, and thus heat resistance
- **Stress adaptation during growth**
  - Stress adaptation can enhance resistance to heat or other stresses

How to control spoilage by fungi (focusing on HRM)?

Mold spoilage cues & potential food safety implications

- **Cues of mold spoilage in beverages and juices**
  - Mold colonies on product surface
  - Floating mycelia – may like cotton ball
  - Product clarification / fruit disintegration (due to pectinolytic enzymes)
  - Swollen package – Some HRM produce CO_2

- **Mold growth may raise pH** (1)
  - 58 species of 21 genera of molds grown on tomato juice for 35 days
  - "All molds except two raised the pH from the initial pH 4.1 to a range from 4.9 to greater than 9.0"
  - "Thirty-three of the Fungi Imperfecti (53%) raised the pH to values above 7.0"

Note: There are multiple studies (2,3) that co-inoculating *C. botulinum* and molds showed *C. botulinum* toxin production

1. Mundt, J.O. 1978. Effect of mold growth on the pH of tomato juice. J. Food Prot. 41:267-268.
2. Draughon, F.A., S. Chen, & J.O. 1988. Mundt. Metabiotic association of *Fusarium*, *Altemaria*, and *Rhizoctonia* with *Clostridium botulinum* in fresh tomatoes. J. Food Sc. 53:120-123
3. Odlaug, T.E & I.J. Pflug. 1979. *Clostridium botulinum* growth and toxin production in tomato juice containing *Aspergillus gracilis*. Appl. Environ. Microbiol. 37:496.

Heat resistant molds (HRM) important to beverage spoilage

- **Definition of HRM?**
  - Not see official definition
  - Usually survive heat shock of **75C/30min**

- **HRM important to thermally processed beverages/foods**
  - ***Byssochlamys, Neosartorya, Talaromyces*** – common
  - ***Eupenicillium, Eurotium, Hamigera, Thermoascus, Rasamsonia, Monoscus*** – less common
  - Species in ***Hamigera*** and ***Thermoascus*** form highly heat resistant ascospore

- **Why HRM is so big a challenge?**
  - **Very heat resistant and can survive common beverage pasteurization processes**
    - Most heat resistant ascospore may exist in nature (not in laboratory)
    - Product quality may suffer if increasing pasteurization temp to fully destroy HRM
  - **Grow at acidic pHs**
    - *Byssochlamys* can grow between 2.0 and 9.0.
    - *N. fischeri* grew well at pH 3.0-7.95.
  - **Some HRM growth under very low oxygen tension** (microaerophilic)
    - *B. fulva, B. nivea, B. spectabilis* - can grow at very low O_2 tensions, producing CO_2.
    - A small amount of O_2 in headspace or slow leakage of O_2 through package can provide sufficient O_2 for growth
    - Production of CO_2 may cause swelling
  - **Not fastidious in nutrient requirement**
  - **Produce enzymes** (e.g., pectinolytic enzymes) that can destroy product quality
  - **Produce mycotoxin**, e.g., Patulin, byssochlamic acid, byssotoxina A, etc, by B. *fulva* or *B. nivea*

How to control fungal, including HRM, spoilage?

Control Ingredients HRM load

- **Ingredients contaminated with soil**
  - Soil, esp. orchard soil – harboring all kind of microorganisms, esp. heat resistant mold (HRM)
  - Detected HRM in plantation soils – palm, sugar cane, rice, cotton, barley, banana, etc.

- HRM in soil: 10 – 100 CFU/10g
- HRM on fruits prior to processing: 10 CFU/100g

- **Ingredients that could be sources of HRM:**
  - **Fruits /fruit juices or puree** from fruits on or near ground
  - **Liquid sweeteners (sucrose, HFCS):** Mold can grow during storage; Known source of heat resistant mold
  - **Dry sugar**
  - **Pectin:** Known source of heat resistant mold
  - **Tea leaves:** Known source of heat resistant mold
  - **Root powders / coconut water**
  - **Spices**
  - **Honey** – both HRM and butyric anaerobes detected

Better to control HRM via ingredient control
because normal pasteurization unlikely inactivates all HRM

- *Byssochlamys* sp.: 5 CFU/100g or even lower could cause spoilage
- Often see ingredient spec on HRM: <1 CFU/100g

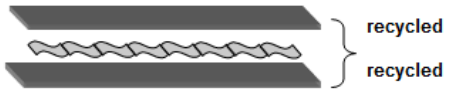
- Frac, M., S. Jezierska-Tys, & T. Yaguchi. 2015. Occurrence, detection, and molecular and metabolic characterization of heat resistant fungi in soils and plants and their risk to human health. Adv. In Agronomy. 132:161-204
- Rico-Munoz, E. 2017. Heat resistance molds in foods and beverages: recent advances on assessment and prevention. Curr. Opinion in Food Sci. 17:75-83

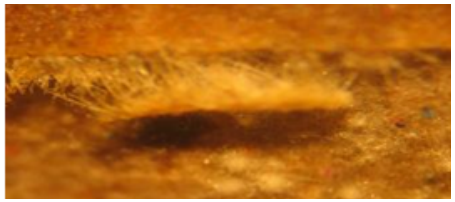
How to control fungal, including HRM, spoilage?

Packaging:

Controlling mold (& HRM) contamination from environment via good GMP can be challenging since so many steps could go wrong

- **Caps**
  - Generally low bioload
  - Not aware of HRM on caps
- **PET bottles:**
  - Could harbor yeast, mold, lactic acid bacteria, etc.
  - HRM could be present, but usually low
    - ➢ So usually test a large # of bottles for HRM
  - Merchant bottles coming on pallets vs on-site blown bottles
- **Bottle pallet tiersheets:**
  - Known source of mold, including HRM
  - Recycled cardboard tier-sheets – esp. worse
  - Cardboard tier-sheets generate dust – source of cross-contamination during bottle handling





- **Paperboard for primary packaging:**
  - Reported: *Penicillium variotii* & *Talaromyces flavus* = 0.71 – 0.35 CFU/100cm^2 & 1 CFU/100cm^2, respectively (1)

1. Frac, M., S. Jezierska-Tys, & T. Yaguchi. 2015. Occurrence, detection, and molecular and metabolic characterization of heat resistant fungi in soils and plants and their risk to human health. Adv. In Agronomy. 132:161-204

How to control fungal, including HRM, spoilage?

Processing environment:

- **Empty bottle receiving /depalletizing /conveying areas**
  - "Dusty" – Controlling "dust" via GMP is challenging
- **Bottle rinse**
  - Air rinse, if not done correctly, could further contaminate the bottles
  - Water rinse – with good quality water and in right spray pattern
- **Wood pallets**
  - Difficult to clean. Could harbor mold and HRM.
- **Cap boxes**
- **Dry ingredient storage area**
- **Dry clean areas**
- **Filling environment:** Micro buildup during long run time, unsanitary design
- **Product cooling water microbial quality**
- **Plant air quality & flow pattern**

How to control fungal spoilage, esp. HRM?

Processing:

- Using low HRM ingredients
- Right sanitation program
- Line hygienic design
- Increase product pasteurization lethality to control HRM – but need to mind product quality
- Chilled storage and distribution for HRM control
- Incorporation of anti-mold preservatives, e.g., sorbate, etc.
- Effective "Inversion"

Effectiveness of “Inversion”

Efficacy of "Inversion" of "Hot Fill & Inversion" Process

- **Purpose of inversion:**
  - To inactivate any **spoilage microorganisms coming from bottles/caps and filling contamination**
  - Likely to inactivate:
    - Yeast vegetative cells
    - Yeast ascospores
    - Mold hyphae
    - Mold conidia
    - Chlamydospores (?)

    Note: Heat resistance varies with physiological state/age
  - Very unlikely to inactivate
    - Mold Ascospores (asci, ascomata)

- **"Inversion" types**
  - Laydown
  - Carmel hump
  - "Twister"
  - "Bumpers" during laydown
  - "Squeezer" for flexible packaging

- **Filling temperature:**
  - Around 175-185F for PET bottles and higher for glass
- **Inversion time:**
  - Varies from several seconds to several minutes depending on line design & package heat tolerance

Note:
- Heat resistance studies – usually done with **rehydrated conidia**
- Mold to be inactivated with "inversion:" could be in **dry state - actual heat resistance unknown**

Bioload from packages & filling contamination

Packaging & Filling /Capping Environment

- **Contaminated packaging materials**
  - Bottles, caps
- **Packaging handling equipment**
  - Depallitizer
  - Bottle conveyors
  - Air rinser
  - Cap handling
- **Air quality of plant**

- **Filling/capping environment**
  - Is filling/capping inside an enclosure?
  - Is air inside the enclosure HEPA filtered?
  - Enclosure overpressure / air flow pattern?
  - Can the enclosure be thoroughly sanitized?
  - Any dead ends?
- **Run time** – too long & allow microbial buildup

GMP (sanitation /cleaning) is critical, but tough to do

Bottle bioload

- **Bottle rinse/decontamination**
  - **Air**
    - For foreign material removal
    - Can it effectively remove organisms?
  - **Water**
    - Water rinsing with optimized flow and spray pattern may allow effective micro. removal
  - **PAA rinse**
    - Inactivate/reduce bottle/cap bioload
  - **Pre-decontamination** of bottles/caps with other means
    - UV treatment
- **Source of bottles:**
  - Merchant bottles vs. Blowing bottles onsite
    - Tightly couple bottle blowing & filling
  - Does blowing process reduce bottle bioload? – probably so

Bottle bioload

Bottle mold bioload study (1):

- Bottles blown on-site vs bottle made from off-site plant

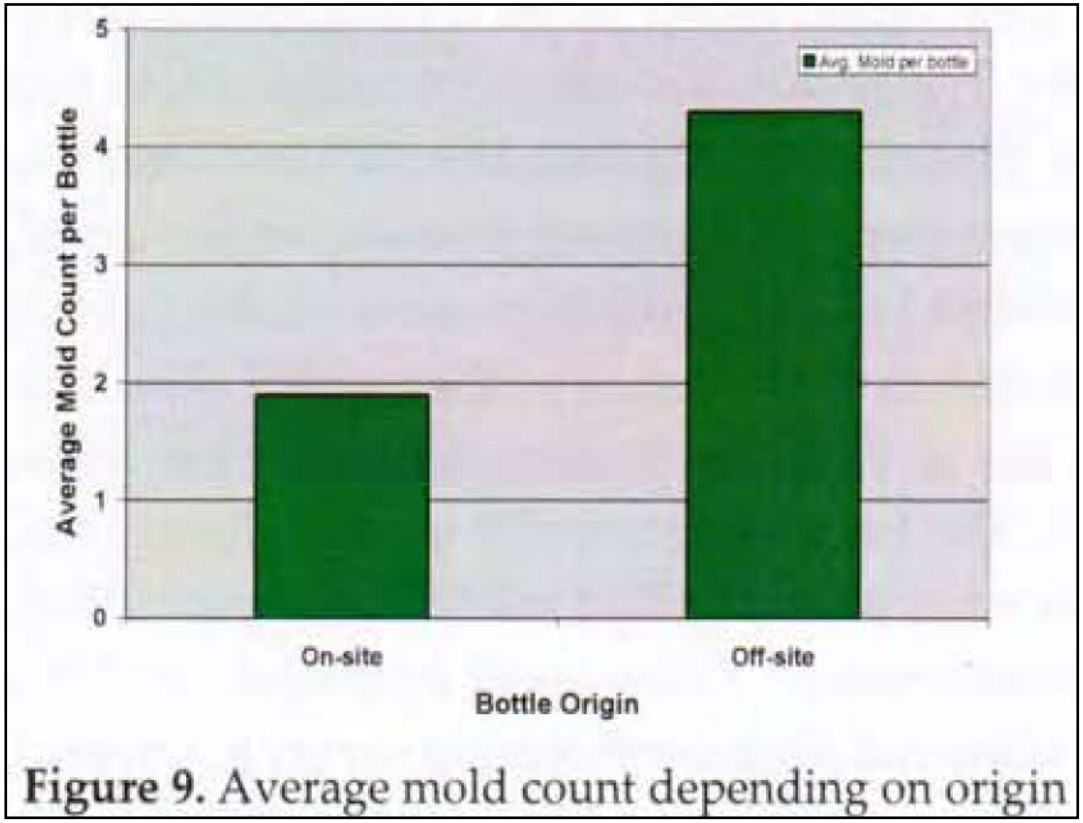


Figure 9. Average mold count depending on origin

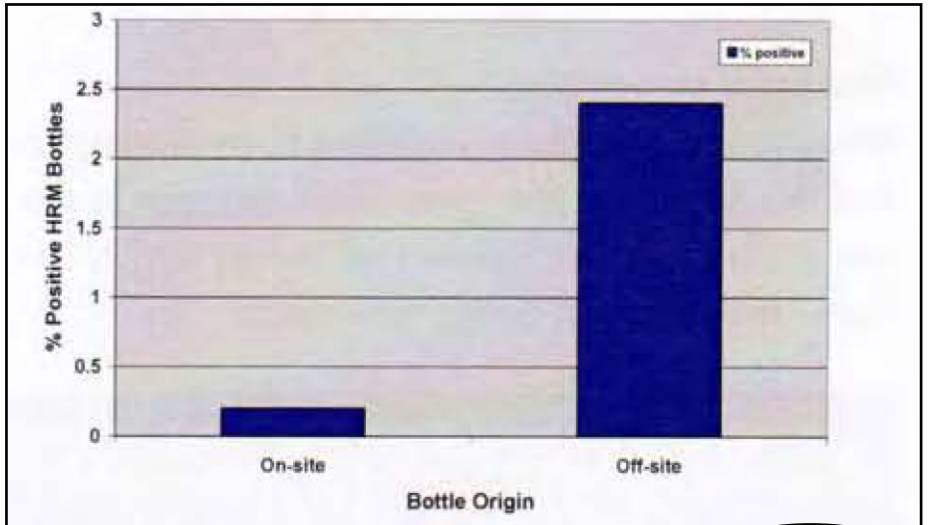


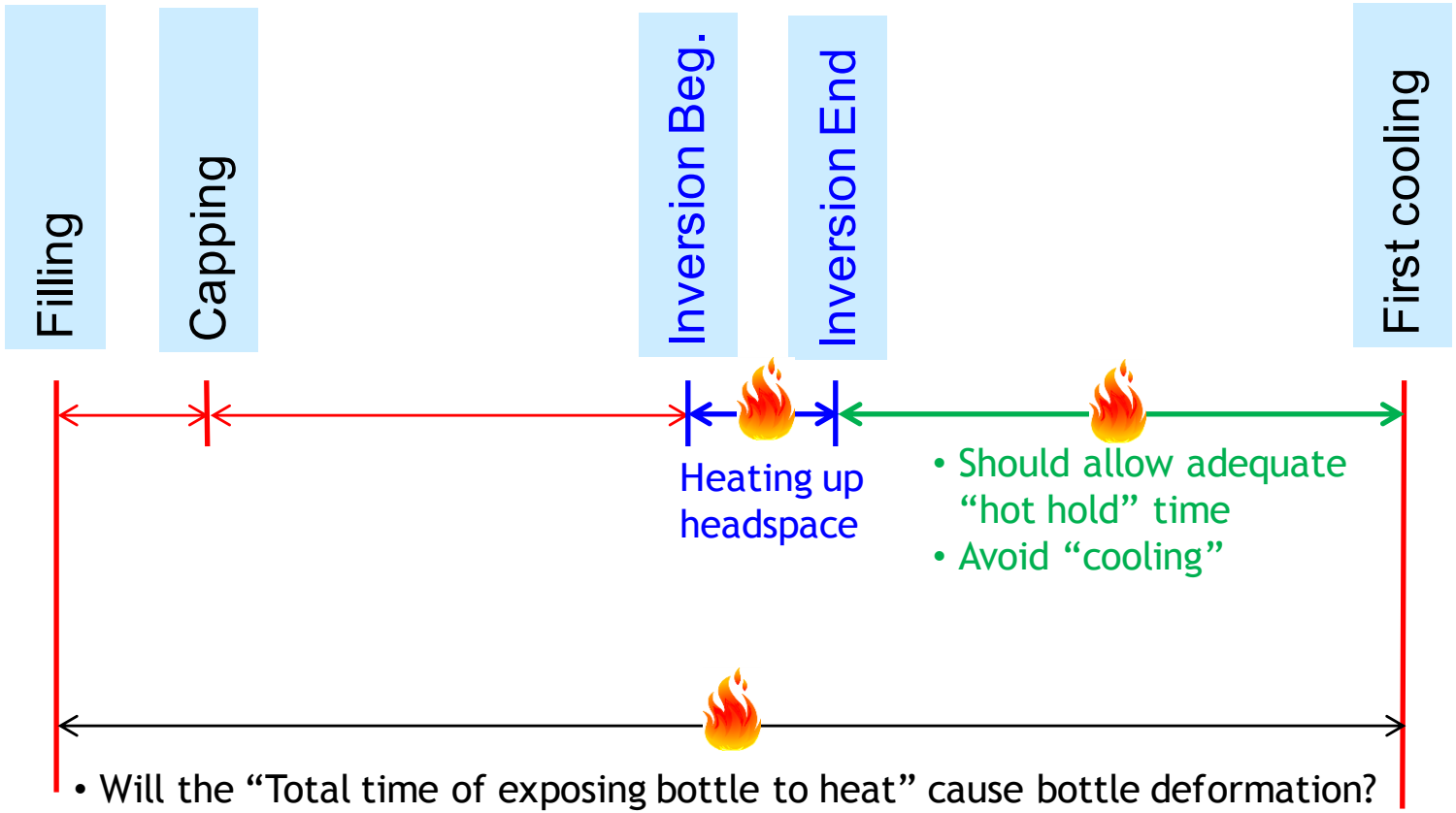
Figure 11. Percent Positive Bottles for *Byssochlamys spectabilis* by the Heat-shocking Method

- 1/537 on-site bottles contained 1 ascospore of *B. spectabilis*
- 4/166 off-site bottles contained at least 1 ascospores

1. Rico, E., S. Johnson, J. Houbraken, & R. Samson. 2007. Sweetners and PET bottles as a source of fungal spoilage of beverage. In "Food Mycology 2007: Emerging Mold Problems and Spoilage in Food and Beverage." By R.A. Samson, J. Dijksterhuis, J. Houbraken, E. Rico, and S. Johnson. Proceedings of Food Mycology, June 7-8, 2007, Key West, USA.

Effectiveness of "Inversion"

- **Important times to consider:**



- Will the "Total time of exposing bottle to heat" cause bottle deformation?
- Spoilage is likely to occur with deformed bottles
  - Balance between bottle decontamination vs. deformation

Factors affecting "hot fill & Inversion" efficacy

Packaging → Packaging handling & treatment → Filling → Capping → Inversion → First cooling

- Packaging materials handling equipment
- Air quality of plant
- Decontamination of bottles/caps prior to filling?

- Filling temp/In-bottle temp
- Fill level (heat transfer to headspace & available headspace O_2)
- Clean filling
  - Bottle neck spray to remove soiling before capping
- Mode of filling
  - Displacement or volumetric?
  - Flood fill (no headspace) may not be the best
  - Impact of viscosity?
- Steaming cap/headspace
- Wet filled bottles – evaporation cooling?
- Microbial levels of filling/capping environment – e.g., inside enclosure

- Duration of inversion (inversion angle to move headspace air bubble)
- Type of inversion
  - Laydown
  - Camel hump
  - "Bumpers" during laydown
  - "Squeezer" for flexible packaging
- Time from inversion to first cooling (i.e., hot hold)
- Additional heating (steaming/hot water shower) prior to cooling

- Contaminated packaging materials

- Cooling water micro quality
- Any bottle deformation before cooling

Other factors affecting "Hot Fill & Inversion" efficacy

- **Product properties:**
  - Brix
  - **Viscosity** – may significantly affect heat transfer to headspace
  - pH / Acidulents
  - Preservatives
  - Fat level
  - Specific heat capacity

- **Bottle design**
  - Bottle temp tolerance (e.g., PET crystallinity)
  - Surface/volume ratio
  - Bottle size/dimensions: Challenge with **small bottles**
    - Short vs **tall bottles**
    - Short vs. **long neck**
    - **Small** vs. big opening
  - "Cold spot" may not always at cap/headspace?

- **Cap design**
  - Size of cap: challenge with **small caps**
  - Any heat "**shielding**"?

- **Plant/line environment:**
  - Surrounding air temp
  - Strong air blow toward bottles
  - Transfer conveyor temp / wetness

Methodology – Determine "Hot Fill & Inversion" log reductions

NFPA report (2000): Optimization of hot-fill-hold processes for juices packaged in PET and glass containers (can be found by "google")

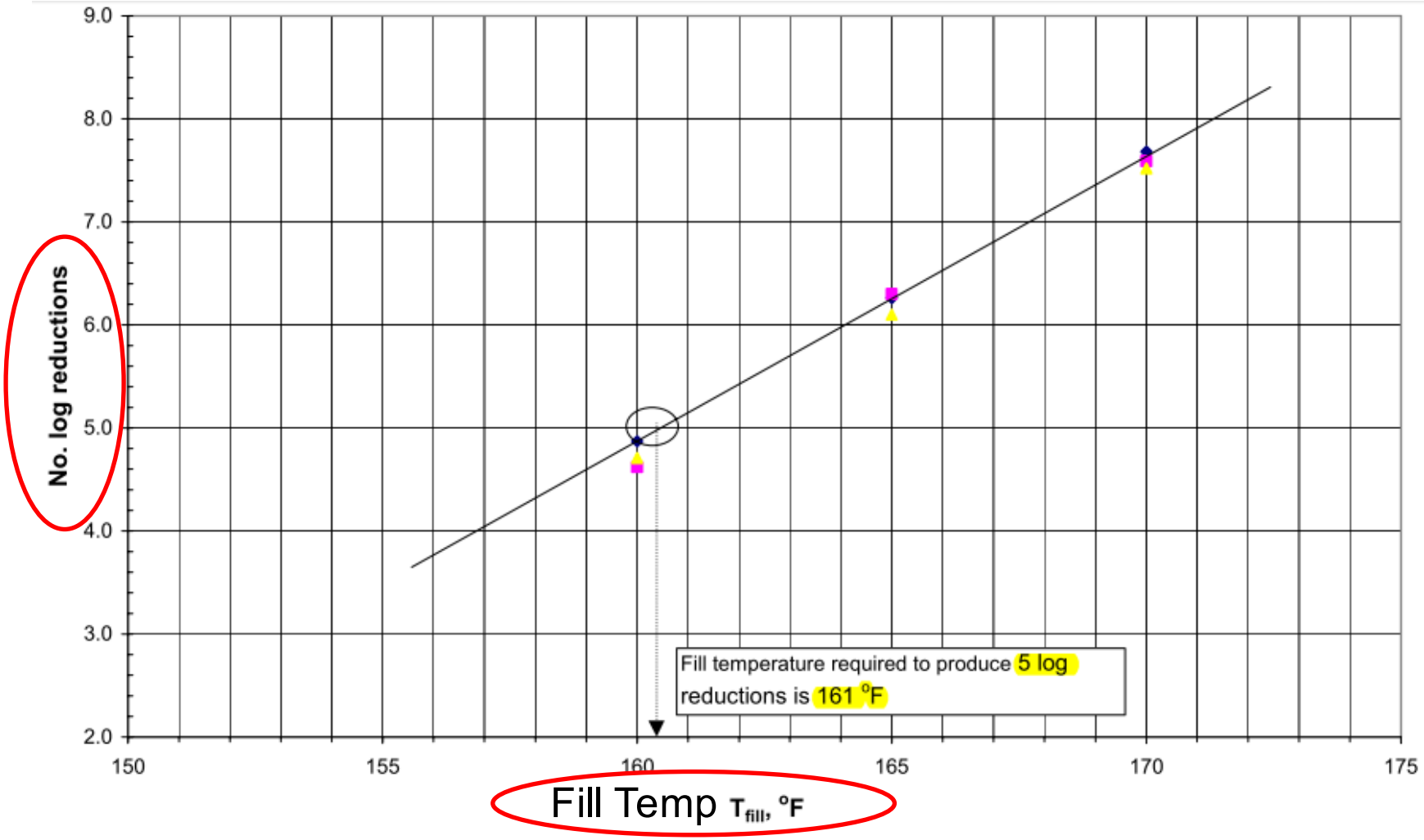


Fig. 4: Example of a fill temperature calculation. The temperature data used was obtained in 1.75 L PET containers held at Horizontal- in-Air condition for a 2 minute hold. *S. cerevisiae* D- and z-values were obtained in grapefruit juice.

Methodology - "Hot Fill & Inversion" log reductions

NFPA report (2000): Optimization of hot-fill-hold processes for juices packaged in PET and glass containers

Steps:

1. Identify the target organism & log reduction – 5 logs of *Saccharomyces cerevisiae* NFPA N-3083 strain was used in the study
2. Conduct TDT study to obtain D and z-values of the target organism
3. Map cold spot inside bottle
4. Calculate cold spot accumulative lethality (log reduction) at different hold times via General method
5. Plot log reductions (cold spot) (at the chosen hot hold time) vs fill temp to get a "log reduction vs fill temp curve"
6. From the curve, get the minimum fill temp (at the chosen hot hold time) required to get the target log kill
7. Conduct challenge study with the chosen target organism to verify adequacy of chosen hot fill/hold process

Table 1: Heat resistance of *Saccharomyces cerevisiae*

Product	D-value (min) at temp (°F) of			z-value (°F)	$D_{150°F}$ (minutes)	$5D_{150°F}$ (minutes)
	135	140	145			
Grapefruit juice	10.2	1.9	1.2	11.7	0.68	3.4
Apple juice	13.0	3.1	1.3	10.8	0.72	3.6
Juice product	9.4	2.8	0.9	10.5	0.52	2.6

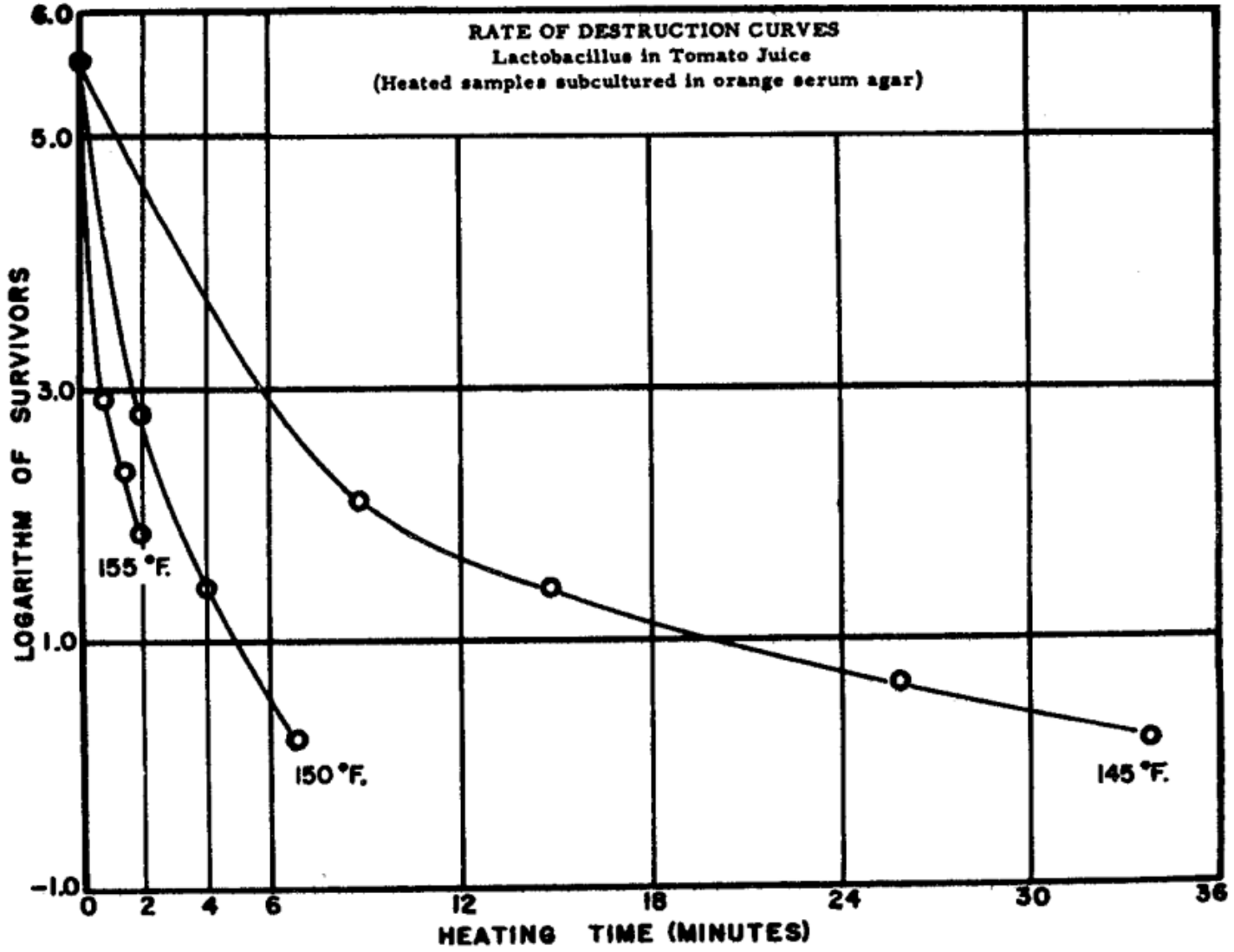
Methodology – Determine what "Hot Fill & Inversion" is adequate

Collier, C.P. & C.T. Townsend. 1954. Container sterilization for acid products by Hot Fill-Hold-Cool Procedures. Proceedings of the Technical Sessions at the 47th Annual Convention of the National Canners Association, Jan 23-27, 1954 (or NCA Information Letter No. 1472)

- Studied 16 lactobacilli & 1 yeast (isolated from spoiled acid products – mainly tomatoes)
- Yeast & lactobacilli in tomato juice & paste:
  - z=10F, F_{150F} = 6.3 - Destroy "100,000 to 1,000,000"
- For organisms "adhering to the inside surface of the can or cover," "they may not come in contact with the product and resistance might be better characterized by that for the phosphate buffer, namely more than 19 min at 150F."
  - One can use F_{150F} = 20 min and z=16F to extrapolate the one of the GMA hot fill inversion tables

Methodology - "Hot Fill & Inversion" log reductions

Collier, C.P. & C.T. Townsend. 1954. Container sterilization for acid products by Hot Fill-Hold-Cool Procedures. Proceedings of the Technical Sessions at the 47th Annual Convention of the National Canners Association, Jan 23-27, 1954 (or NCA Information Letter No. 1472)

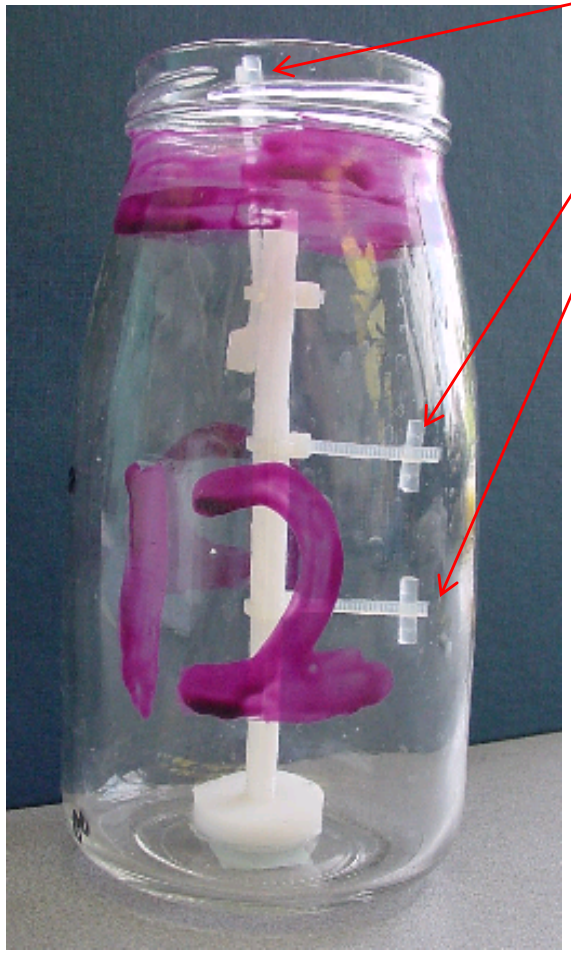


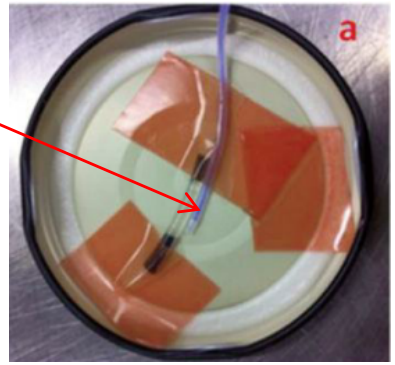
Methodology - "Hot Fill & Inversion" log reductions

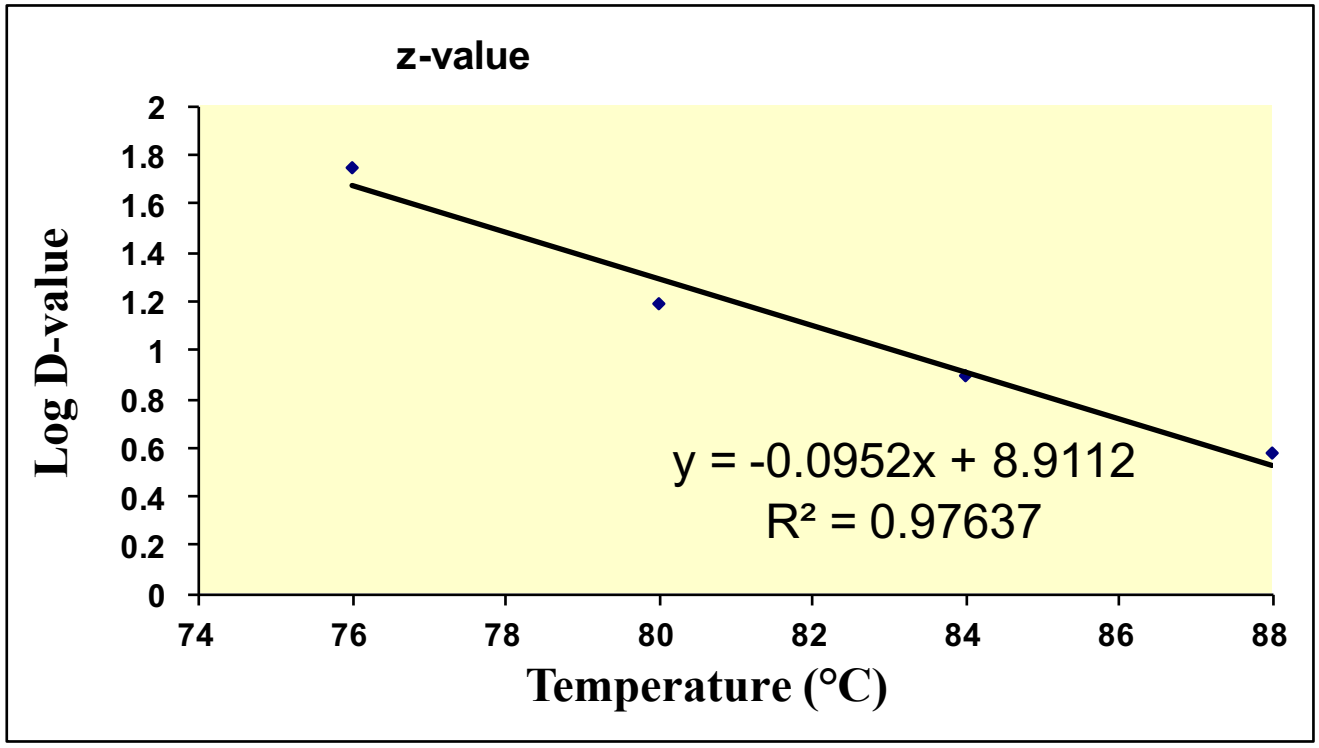
Time-Temperature Indicators (TTI) based on α-Amylase inactivation

- Campden & Chorleywood Food Research Association (CCFRA) published a serious of papers on this

TTI







Other factors affecting “Hot Fill & Inversion” efficacy

Unclean filling:

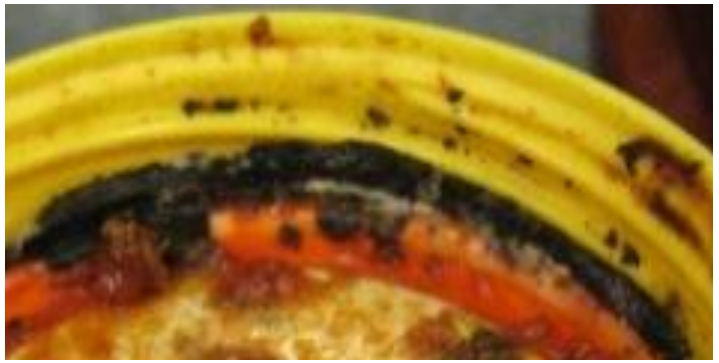
- Splash / spill
- Foaming
- Overfilling
- Soiling Seal/Thread Area

could result in:

- “Caked-on”
- Weakened Seal
- Safety button raised
- Loose cap
- Leak
- Low headspace (esp. with overfill)
  - Low vacuum (safety button pop)
  - Loss of vacuum during storage
  - Spoilage

Other factors affecting “Hot Fill & Inversion” efficacy

Unclean filling could lead to a variety of consumer complaints



Other factors affecting "Hot Fill & Inversion" efficacy

Some of the following complaints could be due to unclean filling

- **Complaints:**
  - "Smoke came out upon opening"
  - "Materials resembling flesh"
  - "Snail"
  - "Jelly like white blob"
  - "Bug under lid"
  - "Worm in threads"
  - "Maggot in the jar"
  - Insects under cap: "larva" / "live eggs" / "lady bug" / "ant" / "Roaches" / "egg nest" / "fly" / "beetles"

Other factors affecting "Hot Fill & Inversion" efficacy

Unclean filling could lead to a variety of consumer complaints

- Mold or other microorganisms growing outside seal
  - Feeding on sauce residues on bottle thread/cap area
- Mold (that growing on product residues in bottle cap area) could traverse seal and get inside bottle
  - FM material complaint (with the FM being mold biomass)
  - "Dead animal" complaint
  - Illness complaints
- Also spoilage of sauce by bacteria/yeast
  - Gassing, fermentation, bubbling/carbonated, wine smell, curdling, etc.
  - Bottle explosion – personal injury

- Insects feeding on sauce residues in thread/cap area
- Bad taste, odor, color, texture
- Bottles/jars hard to open

Other factors affecting "Hot Fill & Inversion" efficacy

- **Cooling water microbial quality** (1):
  - APC < 100 CFU/ml
  - See the referenced paper for more info on importance of cooling water microbial quality

1. Podolak, R., W. Stone, & D.G. Black. 2010. Retort cooling water bacteriological load and possible mitigation strategies for microbial buildup in cooling water. Food Prot. Trends 30:160-167.

Presentation

On-going monitoring - fungal spoilage

Monitor consumer spoilage complaints

- **On-going monitoring of fungal spoilage & continuous improvement**
  - **Consumer spoilage complaints** – micro ID
  - **Consumer spoilage complaint rate**
    - Plot data based on date of complaint received vs spoilage sample manufacturing dates
    - Monthly plot
    - Plot based on plants / lines /product flavor, etc.
  - **Spoilage episodes** (e.g., spoiled lots, market withdrawal)

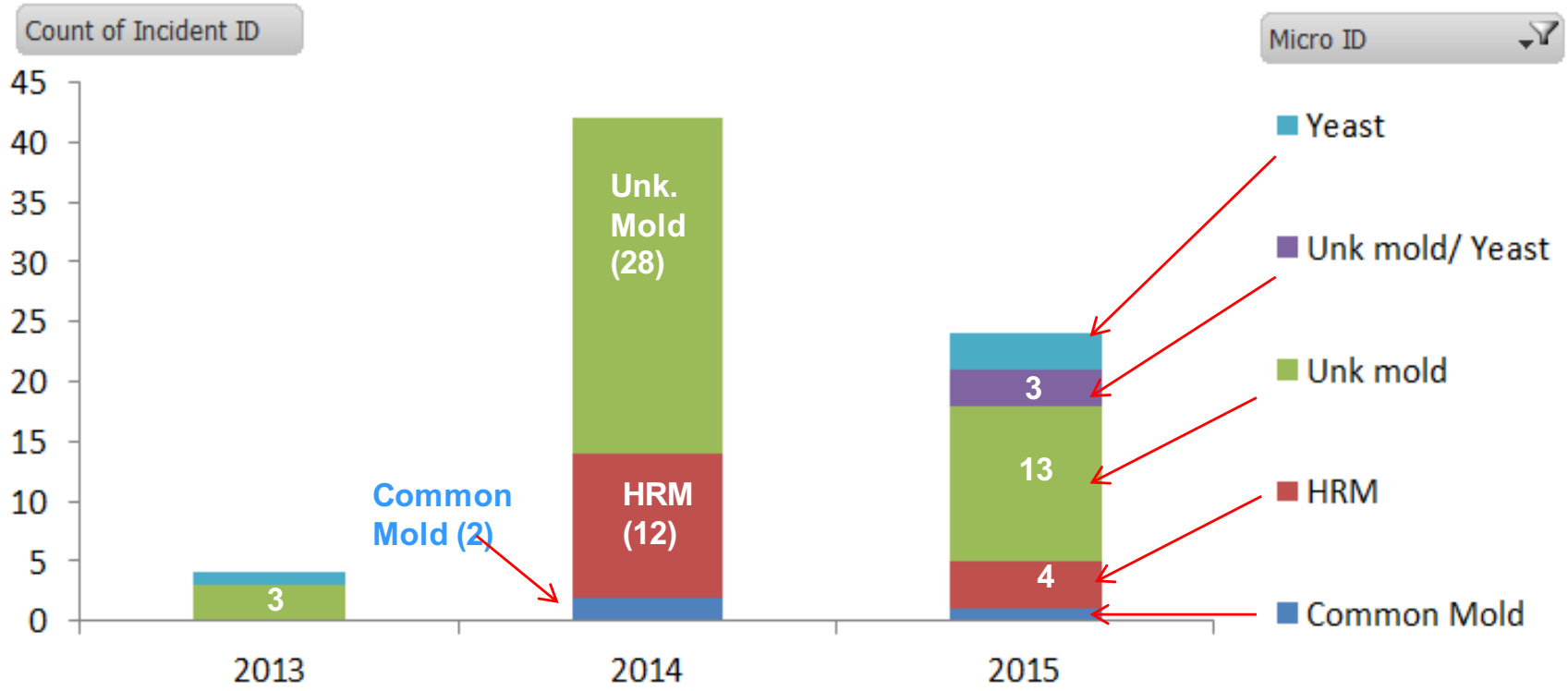
    Ex. spoilage in warehouse / mass spoilage on market
  - **Out-of-spec ingredient**
  - **Out-of-spec air micro, GMP, sanitation, etc.**

Retrieved Spoilage Complaint Samples - Spoilage Identification

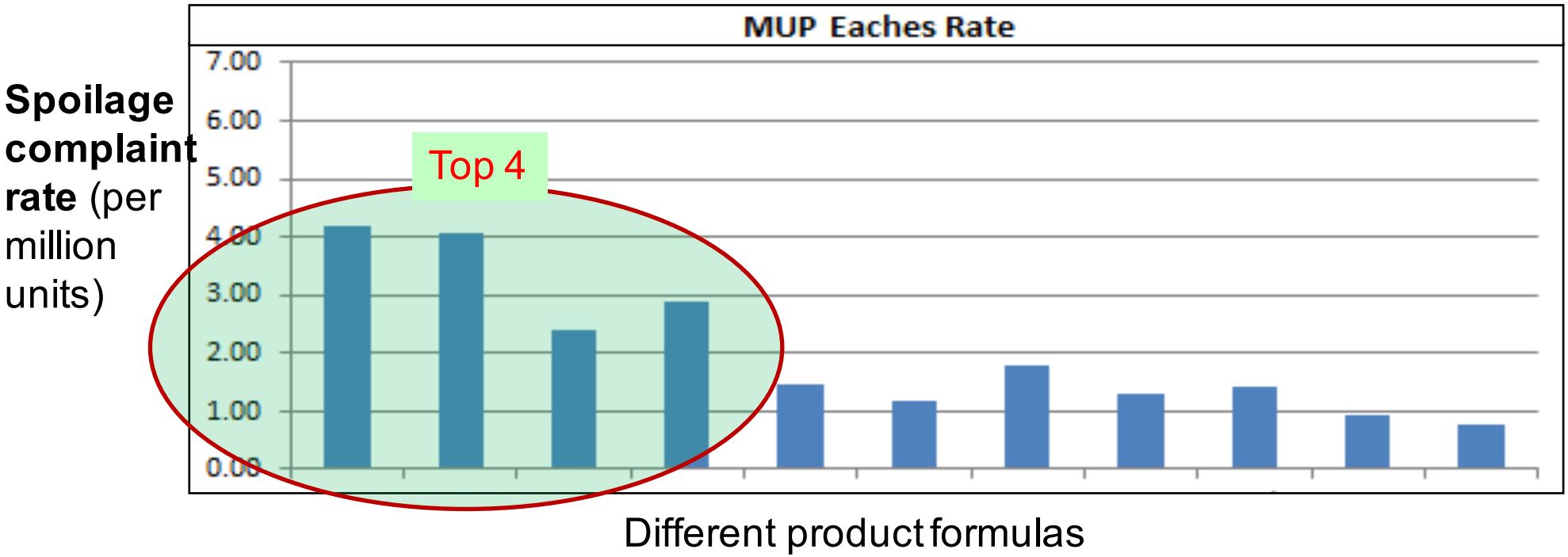
Micro ID data - spoilage samples retrieved from consumers

- **HRM isolated:**
  - *Byssochlamys spectabilis /Paecilomyces variotii*
  - *Byssolchlamys laguncularіae*
  - *Byssochloamys sp.*
  - *Unidentified HRM*

- **Common mold:**
  - *Penicillium spp.*
  - *Aspergillus niger*
  - *Un-named common mold*



Spoilage Consumer Complaint Rates – Impact of product pH & sugar level



Top 4:

- Higher sugar content
- Higher pH

Note: "Number of Consumer Complaint" - based on date of receiving the complaint

Summary

- One fungus may assume different forms of existence, which may have different resistance to stresses.
- Fungi deal with stresses by accumulation of compatible solutes, stress proteins, and forming resting cells.
- By understanding ways of fungal dispersal, we can better understand airborne fungi, etc.
- Ways to control spoilages caused by fungi, esp. HRM, are discussed.
- Effectiveness of "Inversion" are affected by many factors.
- On-going monitoring of fungal spoilage is critical to drive process control and improvement.

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