Molecular Identification And In Vitro Evaluation Of Fungal Growth From Sweet Brioche-like Products

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ABSTRACT

Sweet brioche-like products (pH 5.8-6.2, a_w 0.83) are considered to be intermediate moisture foods, which are significantly susceptible to fungal spoilage and sensory deterioration. Purpose of the study was the molecular identification and determination of *in vitro* growth potential of fungi isolated from filled sweet bread products and their raw materials. Freshly produced praline-, biscuit- or strawberry- filled brioche or raw materials (flour and fillings) were daily supplied by the manufacturer and analyzed immediately or during their shelf-life (20-37ºC, 50-60 days) for fungal presence. A total of 102 fungal isolates were molecularly identified by sequencing the internal transcribed spacer (ITS) region. *In vitro* fungal growth of selected isolates (42 out of 102), representing the major fungal genera identified, was assessed with spot inoculation on Malt Extract Agar of pH 6.2 (adjusted with 5 N NaOH) and a_w 0.99 (without glycerol) and 0.83 (adjusted with 38% v/v glycerol). The low a_w was studied to imitate the respective values of sweet brioche-like products as well as fillings. Fungal growth was monitored at 25ºC (recommended temperature by the industry) and 37ºC (as temperature abuse) by measuring radial diameter for *max*. 60 days. More than 65% of fungal isolates was identified as *Penicilium* sp., followed by 20% of *Cladosporium* sp. and 7% of *Aspergillus* sp. *In vitro* growth assessment revealed that high a_w permitted the growth of all isolates (*n=42*) at 25ºC, whereas only 33% had a growth potential at 37ºC, most of which belonged to genus of *Penicillium*. On sweet brioche-like media (a_w 0.83), no growth was observed at all isolates except for three *Aspergillus* sp. isolates at 37ºC. Contrary to temperature abuse conditions, more than 80% of the isolates, which included all the identified genera, were able to grow at 25ºC and a_w 0.83, highlighting the potential visual deterioration of brioche products during their shelf-life. Results of the present study could contribute to the identification and minimization of the risk of fungal spoilage in sweet brioche-like products .

OBJECTIVE

Molecular identification and determination of *in vitro* and *in situ* growth potential of fungi isolated from filled sweet brioche-like products and their raw materials

MATERIALS AND METHODS

A. Isolation & Characterization of Fungi

- Samples:
  - ❑ Freshly produced products filled with cream (praline, biscuit, strawberry) were analyzed:
    - o upon reception (fresh products)
    - o following storage (shelf-life of 55 days) at 20, 25, & 37ºC
  - ❑ Raw materials (flour, enhancer, fillings)

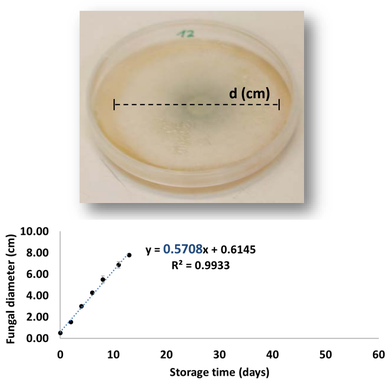
- Fungal isolation:
  - Analysis of samples (10g in 90mL ¼ strength Ringer's solution)
  - ↓
  - Plating on Rose Bengal Chloramphenicol Agar (RBC, 25ºC/ 5 days)
  - ↓
  - Transfer of **single fungi isolate** on Malt Extract Agar (MEA, 25ºC/ 7 days)
  - ↓
  - Collection of fungal spores in 0.1% v/v Tween 80

- Fungal molecular characterization:
  - Inoculation of Malt Extract Broth with 10μL fungal spore suspension (25ºC/ 48-72h)
  - ↓
  - Collection of fungal mycelia
  - ↓
  - Extraction of fungal DNA
  - ↓
  - Fungal identification based on ITS (Internal transcribed Spacer) sequence (primers ITS1-ITS4)

B. *In vitro* assessment of growth potential

- Microorganism: 42 fungal isolates representing the main genera identified.
- Nutrient substrate: Malt Extract Agar of pH 6.2 (adjusted using NaOH) and a_w of:
  - ❑ 0.99: optimum for fungal growth and
  - ❑ 0.82-0.83 (adjusted with glycerol, ca 38% v/v): corresponds to a_w of the product.

Spot inoculation: 10^3 fungal spores (10μL) (N=3)
↓
25ºC and 37ºC for up to 60 days (corresponding to shelf life of the product)
↓
Fungal growth was monitored via radial diameter
↓
Growth rate (μ) corresponded to the slope of the curve of radial diameter (d) against storage time



C. *In situ* assessment of growth potential

- Microorganism: Mix of five (5) *Penicillium* sp. isolates (that demonstrated the highest growth potential in product imitating a_w) (Fig. VII)

Spot inoculation: 10^2 fungal spores (2μL) (N=5) → Inoculation at different sites (upper, bottom, chocolate chip, filling) → Packaging in 20% CO_2 - 80% N_2 → 25ºC & 37ºC for 60 days → Monitor fungal visible growth

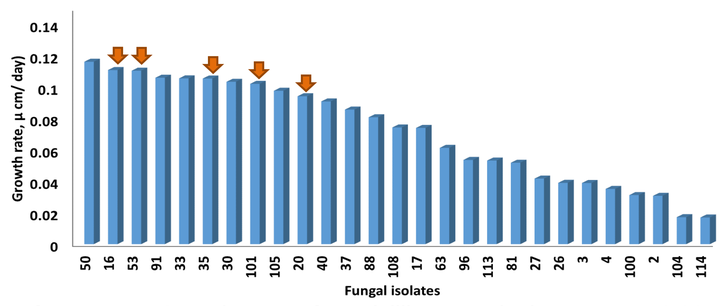


Figure VIII. *Growth rate of Penicillium sp. isolates at a_w 0.82 and 25ºC. Arrows indicate the isolates used in subsequent in situ experiments.*

RESULTS

A. Isolation & Characterization of Fungi

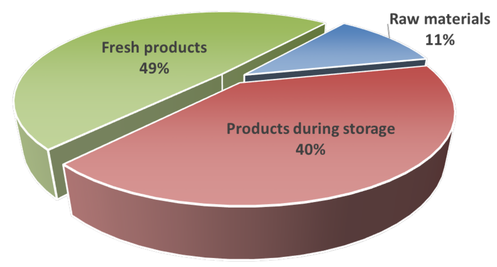


Figure I. *Percentage of fungal isolates (n=102) per origin (fresh products, storage samples, raw materials).*

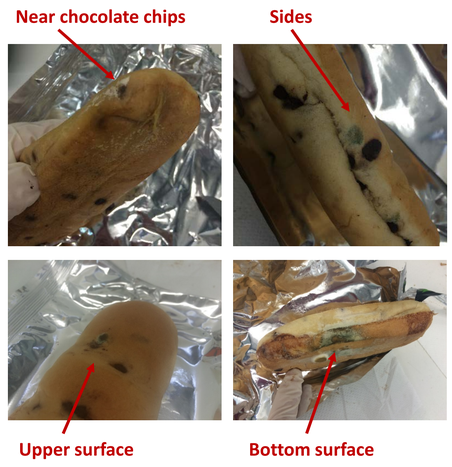


Figure II. *Localization of visible fungal growth in final sweet brioche-like products.*

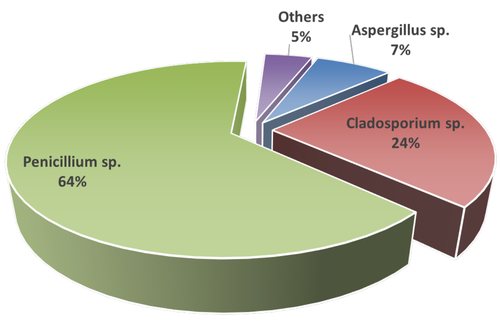


Figure III. *Distribution of fungal isolates (n=102) in main genera identified.*

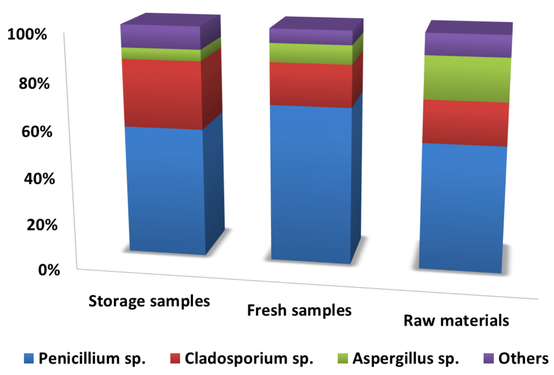


Figure IV. *Distribution of main fungal genera identified across the different isolation sites.*

B. *In vitro* assessment of growth potential

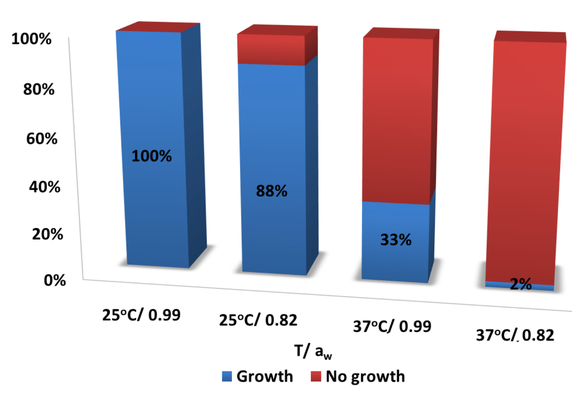


Figure V. *Percentage of fungal isolates demonstrating growth/ no growth under different combinations of temperature and a_w.*

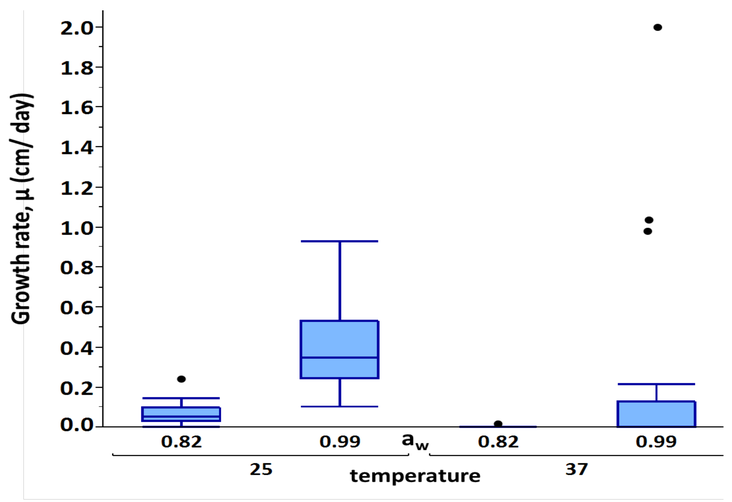


Figure VI. *Heterogeneity in growth potential of fungal isolates under different combinations of temperature (ºC) and a_w. Box plots show the interquartile range (IQR) for all fungal isolates per T/a_w, while horizontal line corresponds to the median.*

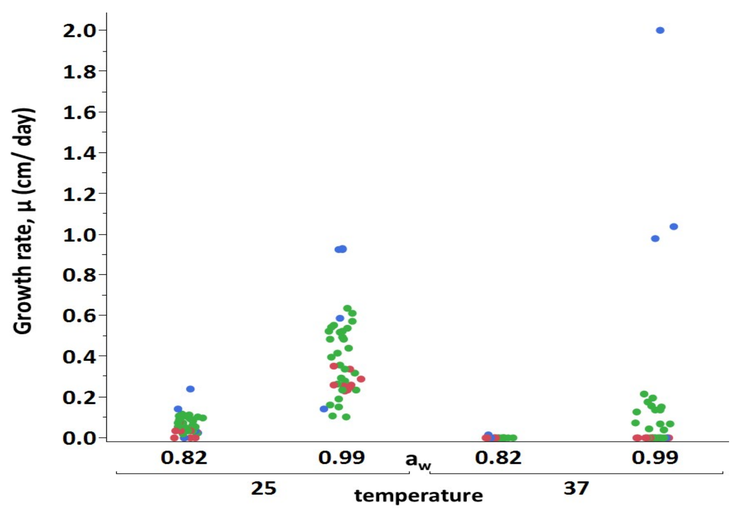


Figure VII. *Intra- and inter- genera heterogeneity in growth potential of fungal isolates under different T(ºC)/a_w combinations. Colored dots represent the growth rates of isolates of Penicillium (green), Cladosporium (red) and Aspergillus (blue) genera.*

C. *In situ* assessment of growth potential

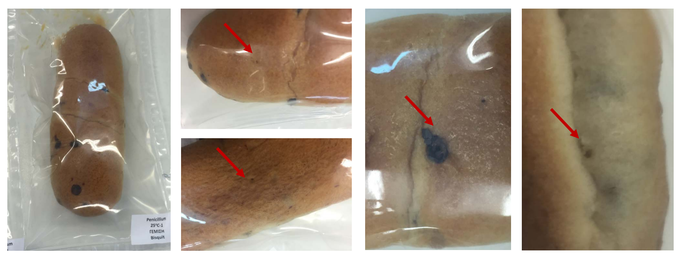


Figure IX. *Sweet brioche-like products with biscuit filling under MAP, after 30 days at 25ºC. Arrows indicate the different inoculation sites on the product.*

- ✓ The majority (89%) of fungi was isolated from final products (Fig. I), while visible fungal growth was observed in discrete sites of the final product and mainly at the end of storage (Fig. II).
- ✓ *Penicillium* (64%), *Cladosporium* (24%) and *Aspergillus* (7%) were the main genera identified (Fig. III) and were similarly distributed across different origin of isolation (Fig. IV).
- ✓ 25ºC allowed the growth of the majority of fungi isolates at product- imitating a_w 0.82, whereas only 33% had a growth potential at 37ºC (a_w 0.99) that belonged to *Aspergillus* sp.(Fig. VII).
- ✓ The highest heterogeneity in growth ability was observed at optimum a_w and T conditions (Fig. VI).
- ✓ Composition of final product, as well as the modified atmosphere successfully inhibited the visible growth of inoculated Penicillia, regardless of inoculum localization or storage temperature (Fig. IX).

CONCLUSIONS

- ✓ The majority of fungal isolates could potentially spoil sweet brioche-like products.
- ✓ Potential abuse temperature could favor the outgrowth of some *Aspergillus* isolates.
- ✓ Combination of hurdles (i.e., preservative, MAP) could successfully control fungal spoilage in sweet brioche-like products.