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# Identification and characterisation of organisms associated with chocolate pralines and sugar syrups used for their production

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ABSTRACT

Spoilage of chocolate pralines, due to growth of microorganisms tolerating low water activity, causes problems in the confectionary industry. Therefore, an increased knowledge on which organisms are present in the chocolate fillings and their tolerance towards low $a_w$, pH, ethanol and other preservatives is needed.

Using media containing 40–50% glucose ($a_w$ 0.872–0.925) bacteria, yeasts and moulds were isolated from chocolate pralines ($a_w$ 0.70–0.898) of nine manufactures and sugar syrups ($a_w$ 0.854) used as ingredient in chocolate praline production by one of the manufacturers. Isolates were identified by conventional microbiological analyses and by sequencing of their 16S rRNA, 26S rRNA (D1/D2-region) or calmodulin genes. Further, for several species the identity was confirmed by amplification and sequencing of additional genes.

In total 677 isolates were identified as belonging to ten different bacteria species, six yeast species and ten mould species with yeast being the most frequently isolated. Bacteria and moulds were found in low numbers, whereas yeast were found in numbers up to $10^7$ CFU/g. The most frequently isolated yeast, bacteria and moulds belonged to the species of *Zygosaccharomyces rouxii*, *Bacillus subtilis* and *Aspergillus terreus*, respectively.

Fifteen isolates were screened for their ability to grow in presence of low $a_w$ (0.65–0.90), low pH (pH = 2.0–7.0), ethanol (0–15%), sorbic acid (0–1500 ppm) and different temperatures (15 °C–25 °C) relevant for chocolate manufacturing. *Z. rouxii* was overall the most tolerant organism to the stress factors and grew within the same range of environmental conditions as found in chocolate pralines. It was able to grow at water activities down to 0.70, ethanol concentrations up to 6.0%, pH down to pH 2.0, sorbic acid concentrations up to 1500 ppm and at all temperatures tested. *Eurotium amstelodami* also showed high tolerance towards all the stress factors except for ethanol. None of the bacteria were able to grow at the conditions tested. However, *B. subtilis* survived the 60 day incubation period.



## 1. Introduction

Spoilage of chocolate pralines, due to growth of microorganisms tolerating low water activity, causes problems in the confectionary industry. Each year 143 000 tonnes chocolate confectionary products are discarded in Europe because of physical–chemical and microbiological spoilage, summing up to an annual cost of 1.2 billion Euros for the chocolate industry (Svanberg, 2012). Therefore, an increased knowledge on which organisms are present in the chocolate fillings and their tolerance towards low $a_w$, pH, ethanol and other preservatives is needed.

Chocolate pralines were originally nuts roasted in caramelized sugar. However, today the definition of chocolate pralines is much broader and covers a large variety of confectionary products (The Nibble, 2012). In the present study, the term chocolate praline refers to a hard chocolate shell with a soft (sometimes liquid) filling. The filling can consist of butter, liquor, sugar, fruit, nuts, marzipan or different kinds of chocolate. The fillings of chocolate pralines contain a high amount of sugar or other dissolved solutes that decrease water activity below levels required to support growth of most microorganisms. In addition, chocolate fillings are often preserved using preservatives such as alcohol, sorbic acid and benzoic acid. Despite this, spoilage of chocolate fillings due to growth of osmophilic yeasts, xerophilic moulds and bacteria that tolerate low $a_w$ cause problems in the confectionary industry. Spoilage is seen as production of off-flavour, slime formation, gas production that leads to cracking of the chocolates or visible growth on the surface of the liquid fraction (Fleet, 1992; Mossel and Sand, 1968).

Problems relating to microbiological spoilage of chocolate pralines were described as early as in the 1920s by Weinzirl (1922) and Hill

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**Table 1**
Microbial counts (colony forming units pr. g, CFU/g) of chocolate praline samples. Identity of yeast, bacteria and moulds is given as percentage of all isolates isolated from the same sample on the specific media. Sample is given as 'manufacturer'-'sample number'. Category is given as A: Fat based filling, B: Sugar based filling, C: Contains marzipan, D: Contains fruit, E: Contains alcohol, F: Contains nuts/coconuts, G: Contains spices, H: Contains coffee.

| | | | | Yeast | | Bacteria | | | | | | | | | | Moulds | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample ($a_w$) | Category | Media | CFU/g | *Zygosaccharomyces rouxii* | *Candida etchellsii* | *Staphylococcus warneri* | *Staphylococcus epidermidis* | *Staphylococcus hominis* | *Kocuria kristinae* | *Micrococcus yunnanesis* | *Bacillus licheniformis* | *Bacillus safensis* | *Bacillus subtilis* | *Bacillus pumilus* group | *Bacillus cereus* | *Penicillium corylophilum* | *Penicillium chrysogenum* | *Penicillium brevicompactum* | *Aspergillus terreus* | *Aspergillus oryzae* | *Aspergillus niger* | *Aspergillus tubigensis* | *Eurotium amstelodami* | *Eurotium repens* |
| 1–1 (N.D.) | A, D | MRS40S | $1x10^3$ | | | | 100 | | | | | | | | | | | | | | | | | | |
| | | PCA40S | $8x10^2$ | | | | | | | | | | | | | 34 | | | | | | | 66 | | |
| | | MY50G | $6x10^2$ | | | | | | | | | | | | | | | | | | | | 100 | | |
| 1–2 (0.852) | A, D | MRS40S | $4x10^2$ | | | | | | | | | | | | | | | | | | 50 | 50 | | | |
| | | DG18 | $9x10^2$ | | | | | | | | | | | | | | | | | | 100 | | | | |
| | | MY50G | $6x10^2$ | | | | | | | | | | | | | | | | | | 100 | | | | |
| 1–3 (0.882) | A, E | MRS40S | $8x10^2$ | | | 100 | | | | | | | | | | | | | | | | | | | |
| 1–4 (0.898) | A, E | MRS40S | $1x10^3$ | | | 83 | 17 | | | | | | | | | | | | | | | | | | |
| 1–5 (0.877) | A, C, F | MRS40S | $9x10^2$ | | | 100 | | | | | | | | | | | | | | | | | | | |
| | | PCA40S | $6x10^2$ | | | | | | | | | | | | | | | 67 | | 33 | | | | | |
| | | DG18 | $1x10^3$ | | | | | | | | | | | | | | | 12 | | 78 | | | | | |
| 1–6 (0.869) | A, D | MRS40S | $9x10^2$ | | | 45 | 55 | | | | | | | | | | | | | | | | | | |
| 1–7 (0.838) | A, E | MRS40S | $7x10^2$ | | | | | 86 | 14 | | | | | | | | | | | | | | | | |
| | | PCA40S | $1x10^2$ | | | | | | 100 | | | | | | | | | | | | | | | | |
| 1–8 (0.763) | A | MRS40S | $3x10^2$ | | | 100 | | | | | | | | | | | | | | | | | | | |
| 2–1 (0.762) | A, C, E | MRS40S | $6x10^3$ | 75 | | | | | | | | | | | | 25 | | | | | | | | | |
| | | PCA40S | $2x10^3$ | | | | | | | | | | 80 | | | 20 | | | | | | | | | |
| | | DG18 | $1x10^3$ | | | | | | | | | | | | | 100 | | | | | | | | | |
| | | MY50G | $3x10^3$ | 100 | | | | | | | | | | | | | | | | | | | | | |
| 2–2 (N.D.) | A, F | MRS40S | $3x10^7$ | 100 | | | | | | | | | | | | | | | | | | | | | |
| | | PCA40S | $5x10^7$ | 100 | | | | | | | | | | | | | | | | | | | | | |
| | | DG18 | $4x10^7$ | 100 | | | | | | | | | | | | | | | | | | | | | |
| | | MY50G | $3x10^7$ | 100 | | | | | | | | | | | | | | | | | | | | | |
| 2–3 (0.721) | A, C, D | MRS40S | $1x10^2$ | | | | | | | | | | 100 | | | | | | | | | | | | |
| | | PCA40S | $4x10^2$ | | | 100 | | | | | | | | | | | | | | | | | | | |
| 2–4 (0.770) | A, C, E, H | MRS40S | $6x10^4$ | 99 | | | 1 | | | | | | | | | | | | | | | | | | |
| | | PCA40S | $5x10^4$ | 100 | | | | | | | | | | | | | | | | | | | | | |
| | | DG18 | $5x10^4$ | 99 | | | | | | | | | | | | | | | | 1 | | | | | |
| | | MY50G | $4x10^4$ | 100 | | | | | | | | | | | | | | | | | | | | | |
| 3–1 (0.753) | A, G | MRS40S | $2x10^2$ | | | | | | | | | | | | | 100 | | | | | | | | | |
| | | PCA40S | $1x10^2$ | | | | | | | | | | | | | 100 | | | | | | | | | |
| | | DG18 | $5x10^2$ | | | | | | | | | | | | | 100 | | | | | | | | | |
| | | MY50G | $3x10^2$ | | | | | | | | | | | | | 100 | | | | | | | | | |