TEMPO® MOST PROBABLE NUMBER TECHNIQUE FOR THE ENUMERATION YEASTS AND MOULDS IN FEED AND FOOD PRODUCTS

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Abstract: The large and diverse group of microscopic foodborne yeasts and moulds includes several hundred species. Both yeasts and moulds cause various degrees of deterioration and decomposition of food. They can invade and grow on virtually any type of food at any time, e.g. they invade field crops such as small grains, nuts, beans, tomatoes, and apples both in the field before harvesting and during storage. They also grow on processed foods and food mixtures. Their detectability in or on foods depends on food type, organisms involved and degree of invasion. Thanks to the research it was possible to evaluate how useful is automated TEMPO® system (bioMérieux) for determining the total number of yeasts and moulds in feed and food products. Twenty artificially contaminated foods were tested including dietetic products, seafoods and dairy products. Further, spices, food additives and animal feed were screened for natural contamination with yeasts and moulds. Statistical analysis of the results by linear regression proves the equivalence of the TEMPO® method and the standard colony count technique, at determination coefficient of 0.934. TEMPO® YM method can be considered as an effective, automated, accurate method for the enumeration of yeasts and moulds in feed and food products. Traditional methods for enumeration of yeasts and moulds in food and feed products involve time – consuming plating techniques.

Key words: microbiological analysis, most probable number (MPN) method, yeasts and moulds

Introduction

The contamination of agricultural commodities with fungi able to produce toxic metabolites is a worldwide concern. Discoloration, quality deterioration, reduction in commercial value and mycotoxic production have been linked to
mouldy contaminated foods and feeds (Pardo et al., 2005). Mould contamination not only generates great economic losses, but also represents a threat to human and animal health, particularly through the synthesis of mycotoxins. Mycotoxins are a structurally diverse group of mostly small molecular weight compounds, produced by the secondary metabolism of fungi, that contaminate the whole food chain, from the harvested products to the plate of consumers (Miličević et al., 2010). Mycotoxins exhibit a wide range of negative effects that the addition of pathomorphological alterations in different organs, exhibit as immunomodulatory, teratogenic, mutagenic and carcinogenic effects (Sinovec and Resanovic, 2005).

Certain foodborne moulds and yeasts may also be a hazard because of their ability to elicit allergic reactions or even cause infections. Although most foodborne fungi are not infectious, some species can cause infections, especially to vulnerable population group, e.g., the aged and debilitated and individuals who are receiving chemotherapy or antibiotic treatment (Semple et al., 1989).

Thanks to the development of numerous automated systems it is possible to examine a lot of samples within relatively short period of time. Instrumental methods are widely used in quantitative analysis, which gives the number of microorganisms that belong to certain group (Russel, 2001).

An automated MPN method, TEMPO®, has been designed by BioMérieux, which can be used for the enumeration of a variety of target organisms including yeasts and moulds. The TEMPO® system is based on a 16 x 3 MPN technique in which a disposable MPN card is automatically filled with a pre-prepared dilution of the sample, together with the TEMPO® reagent. The plastic card consist of three rows of 16 wells each, with a well volume of 225 µL in the first row, 22,5 µL in the second row and 2,25 µL in the third. This difference in volumes effectively achieves a tenfold dilution between one row and the next (Owen et al., 2010).

The yeasts and moulds present in the card reduce the substrate in the culture medium during incubation and cause a fluorescent signal to appear, which is detected by the TEMPO Reader. Depending on the number and type of the positive wells, the TEMPO system calculates the number of yeasts and moulds present in the original sample according to a calculation based on the MPN method (TEMPO® YM 2010).

Current method for enumeration of yeasts and moulds include the International Organization for Standardization (ISO) method. These conventional methods employ agar plates that are incubated for 5-7 days (120-168 h) before obtaining results (Feldsine et al., 2003).

The purpose of this work was to evaluate the TEMPO® YM method for enumeration yeasts and moulds in feed and food products in comparison with standard laboratory method, namely the ISO 21527.
Materials and Methods

**Bacterial strains and growth conditions.** *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763 and *Penicillium aurantiogriseum* ATCC 16025 were used to inoculate food samples. They were grown on dichloran rose bengal chloramphenicol agar (Merck, Darmstadt, Germany) at 25°C for 5 days. Inocula for the sensitivity study were grown in Sabouraud maltose broth (Torlak, Belgrade, Serbia). Serial tenfold dilutions of each organism were prepared in peptone water and the counts were enumerated using the pour plate technique.

**Analysis of artificially spiked samples.** A 10⁻¹ dilution of each food sample (dietetic products, seafoods and dairy products) was prepared by weighing out a 10 g aliquot of the sample and diluting with 90 mL of primary diluents (buffered peptone water or sodium citrate solution). The samples were then homogenized in a stomacher bag with a lateral filter which are especially designed to make easier pipeting of the filtrate after the blending. Furthermore, the samples were artificially spiked with *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763 and *Penicillium aurantiogriseum* ATCC 16025, by adding 1 mL of a 10⁻³ and 10⁻⁴ dilutions of cultures to 10 g of food.

Total yeasts and moulds were enumerated by the TEMPO® and also by colony count technique.

**Analysis of natural contaminated samples.** In this paper we analyzed three different food categories (88 feed and food products), such as spices, food additives (characterized by a high concentration of dyes) and animal feed. Animal feed included soybeans, corn, sunflower meal and other organic feed components. A 1:10 primary dilution was prepared for each sample using TEMPO stomacher bags. Depending on the level of contamination, decimal dilution were performed in maximum recovery diluent.

**Colony count technique.** Surface – inoculated plates are prepared using a specified selective culture media – dichloran 18 % (mass concentration) glycerol agar (Merck, Darmstadt, Germany). Depending on the expected number of colonies, an initial suspension or decimal dilution of the sample / suspension are used.

The plates are then aerobically incubated at 25°C ± 1°C for 5 to 7 days. During this incubation time overgrowth of some yeasts and moulds could have caused an underestimation of counts in some plates.

**Statistical analysis.** Values obtained from the counts were converted to logarithmic form. Results, which were within the enumeration range for both methods, were subjected to correlation coefficient and linear regression analysis.
Results and Discussion

The results, obtained after application of the colony count technique and the TEMPO® method, were analysed statistically by linear regression. The linear regression can be described by the following formula: \( Y = A + B \times X \), at a confidence level 95%.

Regression analysis of artificially spiked samples is detailed in Figure 1. TEMPO® method and colony count technique gave similar results with the coefficient of determination \( R^2 = 0.934 \), an intercept equal to 0.11 and a slope of 0.969. All results were determined as within-range data by both methods.

![Figure 1. Linear regression of colony count technique (log CFU/g) vs TEMPO® YM (log CFU/g) in artificially contaminated samples.](image)

For naturally contaminated samples, results were evaluated with respect to product group. All results were determined as in range data by TEMPO® YM, but three results obtained from spices samples using colony count technique were determined as below-range data (< 10 CFU g\(^{-1}\)) (Table 1). Below-range data were not compared in terms of log\(_{10}\) value.
Table 1. Mean counts and numbers of samples in range data of DG 18 medium and TEMPO® YM for the naturally contaminated samples.

<table>
<thead>
<tr>
<th>Number of samples</th>
<th>Mean counts</th>
<th>I n range data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DG18</td>
<td>TEMPO YM</td>
</tr>
<tr>
<td>Naturally contaminated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spices</td>
<td>30</td>
<td>2.69 ± 0.61</td>
</tr>
<tr>
<td>food additives</td>
<td>26</td>
<td>2.01 ± 0.54</td>
</tr>
<tr>
<td>animal feed</td>
<td>32</td>
<td>3.74 ± 0.76</td>
</tr>
</tbody>
</table>

Out of three type of analysed products, the most contaminated was animal feed. The obtained results indicated that animal feed contained the amount of yeasts and moulds equal to $2.0 \times 10^3$– $9.2 \times 10^3$.

**Conclusion**

The analysis of samples, carried out with the use of the instrumental TEMPO® YM method was proved to be compatible with the colony count techique. Also, the compatibility of the manual and instrumental methods was observed in cases of other microorganisms, such as *E. coli* (Torlak et al., 2008), *Enterobacteriaceae* (Owen et al., 2010) and total number of mesophilic microorganisms (Kunicka, 2007).

TEMPO® YM method can be considered as an automated, accurate, cost – efficient, rapid alternative method for the enumeration of yeasts and moulds in feed and food products offering significant advantages to the reference method. Also, the rapid results enables food manufactures to release their products into the supply chain much earlier than when testing with traditional methods.

**Tempo® tehnika najverovatnijeg mogućeg broja za enumeraciju kvasaca i plesni u stočnoj hrani i namirnicama**

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**Rezime**

Plesni se obično javljaju u kombinaciji sa kvascima i bakterijama i predstavljaju glavni uzrok za povlačenje namirnica iz prodaje. Mnoge plesni ne predstavljaju opasnost po zdravlje ljudi, ali neke proizvode mikotoksine koji su
toksični, kancerogeni, mutageni, ili teratogeni za ljude i životinje. Kvasci su prouzrokovali kvara različitih životnih namirnica, ali većina njih nije štetna po ljudsko zdravlje.

U ovom radu, analizirani su veštački i prirodno kontaminirani uzorci stočne hrane i namirnica kvascima i plesnima. U cilju ispitivanja regresije i koeficijenta determinacije, ukupno je ispitano 20 veštački kontaminiranih uzoraka mlečnih proizvoda, dijetetskih namirnica i morskih plodova. Uzorci su potom analizirani automatizovanoj TEMPO® tehnikom najverovatnijeg mogućeg broja i klasičnom mikrobiološkom metodom prema standardu ISO 21527. Dobijeni koeficijent determinacije od 0,934 ukazuje da je instrumentalna TEMPO® tehnika kompatibilna sa klasičnom mikrobiološkom tehnikom brojanja kolonija. Dobijeni rezultati za obe metode bili su unutar mernog opsega.

Potom je određivan broj kvasaca i plesni u tri tipa prirodno kontaminiranih proizvoda, kao što su začini, aditivi i stočna hrana.

Od tri tipa analiziranih proizvoda, uzorci stočne hrane imali su najveći stepen kontaminacije koji je bio od $2.0 \times 10^3 - 9.2 \times 10^3$. Osim toga, klasičnom mikrobiološkom analizom tri uzoraka začina, dobijeni su rezultati koji nisu bili unutar mernog opsega ($< 10$ CFU g$^{-1}$).

Prednosti primene automatizovane TEMPO® YM tehnike su njegova brzina, tačnost i ekonomska isplativost. Zahvaljujući brzini dobijanja rezultata, proizvođači hrane mogu u vrlo kratkom vremenskom periodu da plasiraju svoje proizvode na tržište.

Literaturni podaci ukazuju da kompatibilnost TEMPO® tehnike i klasičnih mikrobioloških metoda postoji i za aerobne mezofilne bakterije, *E. coli* i *Enterobacteriaceae*.

**References**


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