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Formation of self-organized periodic patterns around yeasts secreting a precursor of a red pigment

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ABSTRACT

Formation of self-organized regular patterns (Liesegang patterns) due to reaction-diffusion process in the gel medium and related to vital activity of yeasts is presented. Two different yeast strains (*Candida pulcherrima* and non-*Candida pulcherrima*) possess a common characteristic feature to secrete a precursor which in the presence of iron(III) ions forms an insoluble red pigment. During yeast cultivation onto solid agar media, periodic spontaneous distinctly spaced red-colored patterns around the yeasts can are formed if the concentration of elemental iron in the growth media is in the range 4–12 mg/L. By changing the composition yeast growth media (YEPD or minimal), growth time and temperature, the mode of yeast inoculation, a variety of red-pigmented patterns around live and proliferating yeasts can be obtained.

1. Introduction

Periodic patterns generated by self-organization are widespread in nature. They are found in rocks and minerals (L'Heureux, 2013), animal or fish coatings (Koch and Meinhard, 1994; Watanabe and Kondo, 2015), bacterial colonies (Itoh et al., 1999; Wakita et al., 2001), human breast (Sis et al., 2004) and kidney (Wood et al., 2013) tissues, human brain (Khonsari and Calvez, 2007) or in plant leaves and blossoms (Davies et al., 2012) and may involve both concentric and stripe patterns, in many cases with curvatures and dislocations.

A wide range of bacteria and yeast strains is capable to produce different types of pigments (Venil et al., 2013; Panesar et al., 2015; Tuli et al., 2015). These microbe-derived pigments are believed to be non-toxic and biodegradable in nature, therefore, they can find applications in various fields such as additives or supplements in food industry, livestock feed, natural dyes or cosmetic products. The production of pulcherrimin pigments by microorganisms as taxonomically unrelated as bacteria *Bacilus cereus*, *Bacilus circulans*, *Bacilus licheniformis*, *Bacilus maceran*, *Bacilus subtilis* (Canale-Parola, 1963; Kupfer et al., 1967; Uffen and Canale-Parola, 1972) and yeast *Metschnikowia pulcherrima* (Sipiczki, 2006; Saravanakumar et al., 2008; Turkel and Ener, 2009) has been reported. When growing in media supplemented with iron(III) salts, these microorganisms are able to produce a water-insoluble red pigment pulcherrimin. Pulcherrimin has been characterized as a ferric chelate or a salt of pulcherriminic acid (2,5-diisobutyl-3,6-dihydroxypyrazine-1,4-dioxide) or a tautomeric form of this compound (Kluyver et al., 1953; Cook and Slater, 1956; MacDonald, 1965). Pulcherriminic acid, a precursor of pulcherrimin, is secreted by microorganisms. Due to reaction with iron(III) ions in the growth medium, red halos around the colonies are formed. Formation of insoluble pulcherrimin depletes iron in the medium and makes the environment not suitable for other microbes that require iron for growth. Therefore, *M. pulcherrima* can serve as a biofungicide in post-harvest disease control (Sipiczki, 2006; Saravanakumar et al., 2008) and in prevention against some bacterial/fungal infections in humans (Turkel and Ener, 2009).

During search for new wild type yeasts with antimicrobial properties, it was found that red-colored pigments were produced on solid iron-containing media around some yeast strains isolated from fermentations of various berries harvested in Lithuania. The formation of the largest amount of a red pigment was characteristic of yeasts identified as *Debaryomyces* spp. (denoted DRV3) isolated from spontaneous fermentations of cherries. The properties of that red pigment coincided with those of pulcherrimin obtained from M. *pulcherrima*; besides, as in the case of

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M. pulcherrima, antimicrobial activity of DRV3 was also dependent on iron concentration (the results of investigation of pigment and antimicrobial properties of DRV3 will be presented elsewhere). DRV3 colonies were also surrounded by red halos and/or accumulated red pigment in the cells when grown on solid media containing iron(III) compounds. Periodic distinctly spaced red-colored rings (Liesegang rings) around the yeasts could be obtained if appropriate conditions were met. The formation of self-organized bands or rings (Liesegang patterns) is widely investigated in inorganic systems where precipitation reaction is coupled with diffusion (Karpati-Swidrowski et al., 1995; Antal et al., 1998; Racz, 1999; George and Varguese, 2005; Msharrafieh and Sultan, 2006; Molnar et al., 2008, 2011; Lagzi and Ueyama, 2009; Badr et al., 2010; Lagzi, 2012; Kalash et al., 2013; Petruska and Barge, 2013; Thomas et al., 2014). One of the precipitate forming reagents is placed as a concentrated solution onto the gel column or in the middle of a gel layer when the experiments are carried out, respectively, in test tubes or in Petri dishes. It diffuses into the gel where another reagent at a much lower concentration is homogeneously dispersed. The chemical reaction between them leads to an insoluble product. Instead of uniform precipitation, alternating rhythmic precipitate and precipitate-free areas perpendicularly to the direction of the diffusion are obtained. The role of a gel is to prevent the convection of solutions and sedimentation of the precipitate. Examples of Liesegang pattern-producing outer/inner electrolyte pairs and gels are, respectively, $AgNO_3/K_2Cr_2O_7$, $NH_4OH/MgCl_2$ $NaOH/MgCl_2$, $KI/Pb(NO_3)_2$, $K_2CrO_4/Pb\ (NO_3)_2$, $K_2CrO_4/CuSO_4$, and polyvinylalcohol, agarose, gelatin. The interest in self-organized patterns arises from the idea to employ diffusion and precipitation reaction as a simple and fast way to produce and control regular structures that can be important in chemistry, physics, biology, materials science and, especially, in medicine as the reaction-diffusion in a bio-gel has was shown to be useful for understanding the formation mechanism of the urinary stones in order to find effective methods to prevent the disease (Xie et al., 2009). Attempts to govern and design regular precipitations were made by applying electric current (Badr et al., 2010) or different pH values (Molnar et al., 2011), by varying concentration and chemical compositions of the gels (Lagzi, 2012) or by controlling concentrations and arrangements of reagents placed onto the gel surfaces (Petruska and Barge, 2013).

The emergence of Liesegang patterns has been observed in a layer of a gel in relation to system of biological origin such as living amoeba *Dictyostelium discoideum* (Kravchenko et al., 2000; Medvinskii et al., 2000). Regular rings appeared as a result of redistribution of folic acid in a solid nutrient medium due to pH decrease in the course of *D. discoideum* cultivation. To the best of our knowledge, regular patterns around yeasts related to their vital activity have not been reported. This paper presents a variety of spontaneous red-pigmented patterns formed around yeast *Candida pulcherrima* (*Metschnikowia pulcherrima)* and DRV3 during their growth on solid agar media. Precursor of a red pigment secreted by yeasts and iron(III) ions in the solid growth media served, respectively, as red precipitate-producing outer and inner reagents. To elucidate whether easily observable formation of red halos and rings could be a model of sediment formation in a biological system, the influence of the composition of growth media, the mode of yeast inoculation, yeast growth time and temperature on pattern development was tested.

Table 1
The mean values of the concentrations of elemental iron in commercial chemicals and prepared yeast growth media determined by ICPOES.

Material	The amount of elemental iron
Agar powder (Reakhim)	100 mg/kg
Agar sheets (Reakhim)	540 mg/kg
Bacto-agar (Difco laboratories)	12.5 mg/kg
Agar-agar for microbiology (Merck)	8.87 mg/kg
Peptone (AppliChem)	43.1 mg/kg
Peptone (Liofilchem)	5.15 mg/kg
Peptone from meat (enzymatic digest) (Merck)	12.56 mg/kg
Yeast extract (Liofilchem)	33.25 mg/kg
Glucose (Applichem)	below 1 μg/kg
Glucose (Biolife)	20.67 mg/kg
Sabouraud	below 1 μg/L
Minimal	6.38 mg/L
YEPD	5.22 mg/L
YEPD	6.21 mg/L
YEPD	8.56 mg/L
YEPD	144.33 mg/L

2. Material and methods

2.1. Chemicals

Agar, peptone, yeast extract and glucose were obtained from various suppliers (Table 1). $FeCl_3{\cdot}6H_2O$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $(NH_4)_2SO_4$ were from Fluka. Biotin, thiamine and β-alanine were from Merck.

2.2. Determination of elemental iron

Elemental iron in chemicals and yeast growth media was determined by inductively coupled plasma optical emission spectrometer (Optima 7000DV, Perkin Elmer, USA) at wavelengths: λ_{Fe} = 238,204 nm; λ_{Fe} = 239,562 nm after microwave assisted sample digestion with $HCl:HNO_3:H_2O$ (1:7:2). The measurements were performed in triplicate.

2.3. Yeasts

C. pulcherrima (*M. pulcherrima*) as a control strain producing pulcherrimin was obtained from the CBS-KNAN Fungal Biodiversity Center, an Institute of the Royal Netherlands Academy of Arts and Sciences (Utrecht, the Netherlands).

Wild type yeast DRV3 producing red pigment was isolated from spontaneous fermentations of cherries that terminated within 7 days and possessed this yeast as dominating. The identification of yeasts was performed using the automatised API 20C AUX (bioMerieux, France) system for clinical yeast identification and applying chemical methods such assimilation of sugars and other substances. Further identification by genetic methods is in progress.

2.4. Growth media

The ability of yeasts to secrete a precursor was tested on three different growth media. YEPD media contained 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 2% agar and various quantities of $FeCl_3$. Minimal growth media contained 2 g/L KH_2PO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L $(NH_4)_2SO_4$, 2 μg/L biotin, 200 μg/L thiamine, 500 μg/L β-alanine, 20 g/L glucose and 2% agar and various quantities of $FeCl_3$.

Commercial Sabouraud medium was purchased from Carl Roth GMBH.

2.5. Yeast cultivation

In order to observe red-pigmented patterns, a strict requirement concerning initial yeast growth should be fulfilled. DRV3 or *C. pulcherrima* initially must be grown on YEPD media containing 2–12 mg/L of elemental iron for 3 days at 25 °C. During the first day of growth, reddish colonies of yeasts should be obtained. After 3 days of growth, white colonies with red halos should be formed