Factors Affecting Growth and Pigmentation of *Penicillium caseifulvum*

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ABSTRACT

Color formation, metabolite production and growth of *Penicillium caseifulvum* were studied in order to elucidate factors contributing to yellow discoloration of Blue Cheese caused by the mold. A screening experiment was set up to study the effect of pH, concentration of salt (NaCl), P, K, N, S, Mg and the trace metals Fe, Cu, Zn, Mn on yellow color formation, metabolite production and mold growth. Multivariate statistical analysis showed that the most important factor affecting yellow color formation was pH. The most pronounced formation of yellow color, supported by highest amount of colored metabolites, appeared at low pH (pH 4). Mold growth was not correlated to the yellow color formation. Salt concentration was the most important factor affecting mold growth and length of lag phase. Production of secondary metabolites was strongly influenced by both pH and salt concentration. The screening results were used to divide the metabolites into the following three groups: 1) correlated to growth, 2) correlated to color formation, and 3) formed at high pH. Subsequently, a full factorial experiment with factors P, Mg and Cu, showed that low P concentrations (2000 mg/kg) induced yellow color formation. Among the factors contributing to yellow color formation, pH and salt concentration are easy to control for the cheesemaker, while the third factor, P-concentration, is not. Naturally occurring variations in the P-concentration in milk delivered to Blue Cheese plants, could be responsible for the yellow discoloration phenomenon observed in the dairy industry.

(Key words: blue cheese, *Penicillium caseifulvum*, pigmentation)

Abbreviation key: a_w = water activity, **HPLC** = High performance liquid chromatography, **PLS** = partial least square regression.

INTRODUCTION

Penicillium caseifulvum is a recently described species isolated from Roqueforti-fermented cheese types, i.e. blue cheese, and their production environment (Lund, 1998). Experiments have shown that metabolites produced by *P. caseifulvum* are responsible for yellowish spots appearing on the surface of these cheeses (Lund, 1995). Although the mold is present and grows in the environment and on the cheese all year round, the discoloration only appears in certain periods, and is most pronounced in the summer. This suggests that unknown factors in the cheese control the production of the colored metabolites. Although discoloration of cheese by contaminating molds has been previously described (Chapman and Sharpe, 1990; Estrup, 1994; Piantanida et al., 1995), knowledge of the biochemical mechanisms and essential factors for discoloration is limited.

Cheese contains all the essential nutrients for growth of fungi (Northolt et al., 1995). However, the proportions of nutrients in the cheese will depend upon the production process, such as coagulation, whey removal, and salting (Martin-Hernández et al., 1992; Coni et al., 1995). As process parameters are held relatively constant when making a specific cheese type, variability in the mineral content of the cheese is mainly due to changes in the milk. In modern dairying, feeding practice is the most important factor for variability, and this is normally linked with season. The greatest seasonal effect occurs in the spring-summer, when cows are fed fresh grass (Grandison et al., 1984; Mariani et al., 1993). Grazing correlates with higher pH in the milk, due to a changed balance of salts (Hermansen et al., 1994). Variability of soluble inorganic phosphorus is the main contributor to changes in the salt balances (Donnelly and Horne, 1986). The concentration of Cu in the milk is lowest in the summer, while Zn and Fe are reported to have the highest level during this time of the year (von Kreuzer and Kirchgeßner, 1990; Larsen and Werner, 1985). Generally, the lowest levels of minerals in the milk occur from April to September (in Denmark), with changes between 5 and 10% (Larsen and Werner, 1984), coinciding with grazing of cows. The

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amount of minerals in vat milk is not standardized prior to cheese making, and as a result mineral levels in blue cheese fluctuate according to the natural variation in the raw milk.

Growth of fungi depends on the composition of the growth media, water activity (a_w), pH, temperature, light, and the surrounding atmospheric gas mixture. The effect of environmental factors on growth of fungi is generally less specific and restricted than the effect on secondary metabolite production. For example, the ranges of a_w and pH within which formation of certain secondary metabolites occur, is more narrow, than the range for conidial growth (Northolt and Bullerman, 1982).

The effect trace elements such as Zn, Cu, Fe, and Mn have on the secondary metabolism of fungi has been investigated (Smith, 1949; Francis, 1985; Griffin, 1994). For example, production of the yellow pigment citrinin, by *Penicillium implicatum*, was induced by addition of Cu and Zn to the growth media (Smith, 1949), and other minerals have been reported to be involved in the formation of other pigments (Francis, 1985).

The purpose of this study was to determine the effect of pH, salt, and selected minerals on the growth and pigmentation of *P. caseifulvum*.

MATERIALS AND METHODS

Organisms and Preparation of Inoculum

Two isolates of *Penicillium caseifulvum* (IBT 15151 ex. Danablu, DK and IBT 15157 ex. Danablu, DK), obtained from the culture collection at Biocentrum-DTU, Technical University of Denmark, originating from two different dairy plants, were used for the experiments. Conidiated cultures were prepared by spreading silica-dried spores on Czapeck Yeast autolysate Agar (Pitt, 1979) with modifications (Samson et al., 1995). Plates were incubated for 7 d at 25°C in the dark, after which colonies were transferred to fresh media and reincubated for 7 d under the same conditions. Suspensions of spores for inoculation were made as 10^6 spores/ml in double distilled water with 0.5% agar and 0.5% Tween-80.

Media Preparation

Media for the experiments were modified from inorganic Raulin-Thom (RT) media, which was modified from Clutterbuck et al. (1932). Salt (NaCl) content and pH of the media was varied from 3 to 10% (w/w) and from 4 to 6, respectively. Adjustment of pH was done prior to autoclaving by adding HCl or NaOH. The minerals varied in the media were P, K, Mg, Fe, Cu, Zn and Mn, and their concentrations ranged from 2000 to

6000 mg/kg (ppm) for P ($(\text{NH}_4)_2\text{HPO}_4$), 500 to 3000 ppm for K (KCl), 150 to 300 ppm for Mg ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.5 to 3 ppm for Fe ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 0.3 to 1.5 ppm for Cu ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 30 to 250 ppm for Zn ($\text{ZnSO}_4 \cdot \text{H}_2\text{O}$), and 0.3 to 10.6 ppm for Mn ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$). All chemicals were from Merck (Darmstadt, Germany), except Zn, which was from Sigma Chemicals (St. Louis, MO). The mineral ranges used correspond to the typical range of these minerals in Danablu cheese 50+ (National Food Agency of Denmark, 1989). All minerals were dissolved in water and added as water solutions to the media. Besides the minerals, all media contained 0.26% ammonium(+)tartrate (BDH Chemicals, Poole, England), 0.26% L(+)tartaric acid (Merck), 5% D(+)glucose (BDH) and 2% agar (B&B, Rødovre, Denmark). Agar, glucose and water were autoclaved in a separate mixture to avoid acid hydrolysis of the agar and Maillard reactions.

Inoculation and Incubation

The prepared media were inoculated with spore suspension in three points with a needle. Two Petri dishes of each medium were covered with adhesive autoclavable cellophane for later evaluation of reverse colony color. Cellophane-covered plates were inoculated with a droplet of 10 μl spore suspension. Fungi were cultured at 25°C in the dark for a 3-wk period.

Experimental Design

A 2-level fractional factorial design ($1/2^5 \times 2^9$) with three center points was constructed for testing the nine factors: pH, salt, P, K, Mg, Fe, Cu, Zn and Mn (Table 1). The design yielded 17 different media. Subsequently, a full factorial experiment (2^3) with three center points was designed to investigate further the factors: P, Cu and Mg. The ranges of P, Cu and Mg in the media were identical to those in the screening experiment while the other factors were held constant at the center point values from the screening experiment, except salt, which was added in the amount of 3% (w/w). The design yielded eight different media. All experiments were designed using the software program Modde version 3.0 (Umetri AB, Umeå, Sweden).

Colony Measurements

The colony diameter was recorded (in millimeters) after 3, 7, 10, and 14 d of incubation. The mean of six colony measurements was used for data analysis. The lag phase was defined as the number of days until the colony diameter exceeded 2 mm.

The color was measured on both the front (obverse) and reverse side of the mold colony. Color measure-

Table 1. Screening experiment for *Penicillium caseifulvum* growth and color formation on media with different pH levels and different concentrations of salt and minerals.

Media	pH ¹	Salt %	Mineral content of the media, ppm (mg/kg)						
			Mg ²	Fe ²	K ³	P ⁴	Cu ²	Zn ²	Mn ²
N1	4.4	3	150	0.50	500	2000	0.3	30	0.60
N2	6.4	3	150	0.50	3000	2000	1.5	250	0.30
N3	4.4	10	150	0.50	3000	6000	0.3	250	0.30
N4	6.4	10	150	0.50	500	6000	1.5	30	0.60
N5	4.4	3	300	0.50	3000	6000	1.5	30	0.30
N6	6.4	3	300	0.50	500	6000	0.3	250	0.60
N7	4.4	10	300	0.50	500	2000	1.5	250	0.60
N8	6.4	10	300	3.00	3000	2000	0.3	30	0.30
N9	4.4	3	150	3.00	500	6000	1.5	250	0.30
N10	6.4	3	150	3.00	3000	6000	0.3	30	0.60
N11	4.4	10	150	3.00	3000	2000	1.5	30	0.60
N12	6.4	10	150	3.00	500	2000	0.3	250	0.30
N13	4.4	3	300	3.00	3000	2000	0.3	250	0.60
N14	6.4	3	300	3.00	500	2000	1.5	30	0.30
N15	4.4	10	300	3.00	500	6000	0.3	30	0.30
N16	6.4	10	300	3.00	3000	6000	1.5	250	0.60
N17	5.4	6.5	225	1.75	1750	4000	0.9	140	0.45
N18	5.4	6.5	225	1.75	1750	4000	0.9	140	0.45
N19	5.4	6.5	225	1.75	1750	4000	0.9	140	0.45

¹The exact pH value of each media was measured after autoclaving and used for data analysis.

²Added as sulfate salts.

³Added as chloride salt.

⁴Added as di-ammonium hydrogen-phosphate salt.

ments of the reverse were done using the cellophane-covered plates. The cellophane was removed, thereby separating the mycelium from the agar surface, uncovering the reverse side of the colony. Color was measured with a colorimeter (Croma Meter, CCR-200, Minolta, Japan) as L*, a* and b* values, which, going from low to high numbers, measures the color spectra as follows: black to white (L*), green to red (a*) and blue to yellow (b*). In order to use values that better described colony colors, L* and b* values were referred to as lightness and yellow color, respectively. Measures of a* were reversed, so the spectra, going from high to low numbers, described red to green, and thus a* was referred to as green color. Measurements were performed after 14 d of incubation. The mean of triplicate measurements was used for data analysis.

Extraction and Analysis of Metabolites

Fungal biomass from 21-d-old colonies (from the screening experiment only) was extracted with a mixture of 50 ml methanol, dichloromethane and ethyl acetate in the proportion 3:2:1 and added 0.5 % formic acid. The organic extract was filtered through a Whatman 1 PS phase separator filter and evaporated on a rotary evaporator to dryness. The residue was dissolved in 1.5 ml methanol and transferred to a vial through a Minisart RC 15 filter (0.45 μ m) before analysis. The extracts were analyzed by reverse-phase high-perfor-

mance liquid chromatography (HPLC) on a Hewlett Packard 1090 series II liquid chromatograph (Hewlett Packard, Germany) with a built-in diode array detector measuring full UV-VIS spectra (200 to 600 nm) for each peak detected at 210 and 340 nm. The gradient ran from 15.0% acetonitrile (Merck 707) in water to 100% acetonitrile, and contained 0.005% trifluoroacetic acid (Merck 633). It was held at a flow rate of 1 ml/min. The column was a 4 mm id $\times$ 100 mm HP Hypersil BDS C-18 (3 μ m particles).

Metabolites were quantified by peak area (milliabsorption units (mAu) $\times$ s) at $\lambda_{\max}$, or maximum absorption (mAu) if interfering substances were found within the area. The peak area of ergosterol in the samples, which is a suitable measure of mold growth (Seitz et al., 1979), was measured and used for standardizing the metabolite content according to biomass units. Retention indices (RI) of fungal metabolites were calculated according to Frisvad and Thrane (1987).

Response Variables and Data Analysis

The response variables in the screening experiment, 17 in all, were the following: mold diameter recorded after 7 and 14 d, the calculated lag phase, color coordinates (i.e. L*, a*, and b* values) for obverse and reverse sides of the colony, and finally eight metabolites were quantified and used for analysis. In the subsequent full-factor experiment, the metabolite extraction was

omitted, and only colony diameter after 14 d and reverse side color coordinates were used for analysis, thus four responses were used for analysis.

The data were analyzed by partial least square (PLS) regression using SIMCA-P for Windows (version 2.1, 1995; Umetri AB, Umeå, Sweden). We previously described the advantages of using this method for physiological characterization of fungi (Haasum and Nielsen, 1998). The statistical principle of the method was described by Wold et al. (1984). The X-matrix for the screening experiment consisted of the design variables given in Table 1. All minerals except P were added to the media as sulfate compounds, and in order to determine the effect of sulfate, it was added to the matrix as an uncontrolled variable (P was added as an ammonia compound to the media, and since this was the only mineral added as such, the effect of ammonia was completely confounded with P).

Prior to statistical analysis, all variables were standardized to mean zero and variance one. In the screening experiment the two *P. caseifulvum* isolates responded alike, thus only one representative is shown in the figures, whereas for the full factor experiments, results for both isolates are shown.

RESULTS AND DISCUSSION

Secondary Metabolites

Seven chromophores represented the majority of the metabolites in all mold extracts. As the examples of

chromatograms for media N10 and N13 show, the seven metabolites were not all present in all extracts (Figure 1). For example, N10 supported low metabolite F production and high metabolite I, whereas the opposite was true for media N13. Metabolite D had two analog forms, which were called D1 and D2. Using results from Frisvad and Thrane (1987) the metabolites B and C were identified as rugulovasine and cyclopeptine, respectively. The chemical structures of the other metabolites (i.e. A, D1, D2, E, F and I) were not identified.

Strong absorption of light was detected for metabolites E and F in the visible wavelength area (380 to 780 nm), indicating that these metabolites were colored products (Figure 2). The spectrum of metabolite F showed especially strong absorption at 350 to 500 nm indicating a yellow to orange-yellow color, while metabolite E absorbed at 350 to 420 nm, indicating a more greenish-yellow color (Figure 2). Metabolites D1 and D2 had slight absorption above 380 nm, indicating that these might have some coloring properties (Figure 2). The remaining metabolites (A, rugulovasine, cyclopeptine and I) did not absorb light in the visible wavelength area.

All metabolites were quantified at the wavelength where peak absorbance was observed. In this fashion, the metabolites A, rugulovasine, cyclopeptine, and ergosterol (used as a biomass indicator) were quantified in chromatograms obtained at 210 nm; metabolites D1, D2, and I were quantified at 335 nm, while E was quantified at 400 nm. Detachment of metabolite F from

Figure 1. Chromatograms of *Penicillium caseifulvum* (IBT 15151) on media N10 and N13 obtained at 254 nm (full line) and 340 nm (dashed line). Metabolites A, B (rugulovasine), C (cyclopeptine), D1, D2, E, F, and I were quantified for statistical analysis (metabolite E was not produced on the media shown here). Norm: Normalized absorption in milliabsorption units.

Figure 2. UV-spectra and retention index (RI) of metabolites from *Penicillium caseifulvum*. Metabolites E and F showed absorption of light in the visible area (380 to 780 nm). mAU: milliabsorption units.

the column was not optimal, causing a broad peak and a span in the retention index. In order to reduce interference from other substances, it was quantified at 432 nm.

Effect of Salt and pH on Growth and Color of *P. caseifulvum*

Growth of *P. caseifulvum* was strongly correlated with salt content of the media. On media with 10% salt,

the average mold colony diameter was 8.4 ± 3.5 mm after 2 wk, whereas on media containing 3% salt, it averaged 38.4 ± 8.8 mm (Figure 3a). Measurements of reverse yellow color (reverse b) correlated well with pH values of the media (Figure 3b). At pH 4 the b average was 44.0 ± 7.7 , while at pH 6 it was 14.0 ± 8.2 . Thus at low pH the reverse colony color of *P. caseifulvum* was more yellow compared with high pH. Production of metabolite F also followed this pattern, so that the

Figure 3. Salt content in the media correlated to measurements of growth (a), and pH correlated to measurements of yellow color (coordinate b on the Chroma Meter) and production of metabolite F (b).

mean production of metabolite F was 5.6 ± 2.0 and 1.2 ± 1.0 mAU $\times$ s on media with pH 4 and 6, respectively. Thus, metabolite F could be used as a measure of yellow color development of *P. caseifulvum* colonies. It should be noted, that all extractions were performed at identical pH values (in 0.5% formic acid), thus the observed correlation between pH and yellow color was not caused by any extra-cellular pH effect.

The PLS model obtained by statistical analysis consisted of four significant components, explaining 70.4% of the variation in the data. Loading component one showed that salt was positively correlated with lag phase, and negatively correlated with growth (colony diameter), and production of rugulovasine, cyclopeptide, and metabolite A (Figure 4a). Thus, high salt content in the media produced longer lag phase, less mold growth, and inhibited production of some metabolites. Extension of lag phase caused by high salt percent is well known for *P. roqueforti* (Godinho and Fox, 1981). Furthermore, high salt-media produced less green and more white colony obverse colors, which agreed with the fact that mold growth was reduced in high salt media, resulting in fewer conidia and consequently less green color.

Loading component two showed the strong negative correlation between reverse yellow color and pH (Figure 4a), which was described earlier. Obverse yellow and reverse lightness were also negatively correlated with pH and thus positively correlated with reverse yellow. This suggests that measuring the colony color of the obverse side of *P. caseifulvum* gives a good indication of the yellowness on the reverse. Visually observed color of the reverse also showed the pH effect, as groupings of yellow and orange-yellow colonies appeared in media with low pH (4), as indicated in the score-plot

(Figure 4b). This observation was notable after 7 d, and very clear after 21 d.

Media pH was also negatively correlated with production of metabolites D1, D2, E, and F (Figure 4a). Thus low pH (4) induced production of these metabolites. Component two also described production of metabolite I, however, contrary to the other metabolites, production of I was stimulated at high pH.

Overall, the model divided the metabolites into three groups: 1) rugulovasine, cyclopeptide and metabolite A, which were associated with mold growth; 2) metabolites D1, D2, E and F, which were associated with low pH and yellow color; and 3) metabolite I, which was associated with high pH values. It is likely that there is a biosynthetic relationship between metabolites D1 and D2 and metabolite F because of the similarities in their UV-spectra (Figure 2) and their loading values in the PLS model (Figure 4a).

Effect of Minerals on Growth and Color of *P. caseifulvum*

All media except N3 supported mold growth. This medium had a pH of 4, 10% salt, low content of Fe (0.5 mg/kg), Cu (0.3 mg/kg) and Mn (150 mg/kg); this combination of ingredients may have caused the lack of mold growth. However, the PLS loading plot showed that the effects of minerals on mold growth, color and metabolite production were small or insignificant (all loading values of minerals were smaller than 0.5) compared with those of pH and salt.

In order to focus on the effect of minerals on color formation, an additional PLS analysis was performed using the colored metabolites E and F and the three reverse color values as response variables. The model,

ment suggest that P is important for metabolism that support mold growth but it inhibits metabolism causing yellow color formation. Thus, high levels of P may inhibit or reduce production of the colored metabolites E and F.

The P-level in milk is reported to drop during the summer season (Larsen and Werner, 1985), coinciding with the period where pigmentation problems mostly occur. In conclusion, addition of phosphorous salts to the cheese-milk may prevent or reduce formation of pigments by contaminating molds. However, it may also increase the basis for mold growth.

In this experiment P was added as $(\text{NH}_4)_2\text{HPO}_4$, and since it was the only mineral added as an ammonia salt the effect of P and N could not be statistically separated. Therefore, future research on this subject should investigate the effect of P and N separately.

Similar to P, high levels of Mg (300 mg/kg) were positively correlated with mold growth and negatively correlated with yellow color formation. In contrast, high levels of Cu (1.5 mg/kg) were positively correlated with yellow color formation. Smith (1949) also showed that *Penicillium implicatum* production of the yellow pigment citrinin was induced when Cu was added to the growth media. However, the effect on yellow color formation was very small within the levels of Cu found in Blue Cheese (0.3 to 1.5 mg/kg). This agrees with the fact that levels of Cu in milk used for Blue Cheese production are highest in the winter, but the yellow color formation is mostly a problem in the summer. Therefore, if high concentrations of minerals initiate yellow color formation in *P. caseifulvum* colonies, minerals other than Mg, Cu, or P are the cause. Zn, for instance, may trigger yellow color formation, because it was positively correlated with yellow color formation (Figure 4a), and it is present in milk in highest levels during the summer (Larsen and Werner, 1985; von Kreuzer and Kirchgessner, 1990). Additionally, high levels of S (520 to 842 mg/kg) were also positively correlated with yellow color (Figure 4a). However, S was not a controlled factor in this experiment.

CONCLUSIONS

Of the chromophores representing the majority of metabolites produced by *P. caseifulvum* in the different media, E and F were identified as the only colored products, i.e. greenish-yellow and orange-yellow, respectively. The production of these metabolites was also correlated with measurements of yellow color on the reverse side of mold colonies as well as visual observations.

Yellow pigmentation of *P. caseifulvum* colonies was induced at low pH (4). Even a very small decrease of

pH (0.1 pH units) in the media caused a significant increase in yellow color development. Thus, these findings stress the importance of controlling the pH in the Blue Cheese process.

Production of the colored metabolites was not affected by salt content or mold growth. Instead, increased salt content caused retardation of mold growth and increased the length of the lagphase.

Of the minerals, P was the only one that affected yellow color formation of the colonies. High levels of P (6000 mg/kg) caused less yellow color development. Thus, the P-level may have a regulatory effect on production of the colored metabolites E and F, and therefore addition of phosphorous salts to the cheese-milk may help prevent or reduce formation of yellow metabolites by *P. caseifulvum* on Blue Cheese. However, high levels of P also increased mold growth, which could limit the effectiveness of P added to the cheese-milk. This study could not identify any significant effects from any of the other minerals studied, i.e. Mg, Cu, Mn, Fe, and S (which was an uncontrolled factor).

Two of the seven chromophores that were analyzed were identified as rugulovasine and cyclopeptine, while the remaining metabolites were not identified. The seven chromophores were divided into three groups. One group consisted of metabolites related to mold growth, these were metabolite A, rugulovasine, and cyclopeptine. A second group consisted of colored metabolites produced at low pH (4 to 5); these were metabolites E, F, D1, and D2 (D1 and D2 were only slightly colored). Finally, a third group, consisting of just one metabolite, was produced at high pH (7); this was metabolite I.

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