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Citation:

FAIRCLOUGH, Andrew, CLIFFE, Dawn and KNAPPER, Sarah (2011). Factors affecting penicillium roquefortii (penicillium glaucum) in internally mould ripened cheeses: implications for pre-packed blue cheeses. International Journal Of Food Science & Technology, 46 (8), 1586-1590. [Article]

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Factors affecting *Penicillium roquefortii* (*Penicillium glaucum*) in internally mould ripened cheeses: Implications for pre-packed blue cheeses.

Andrew C. Fairclough^{a*}, Dawn E. Cliffe^a and Sarah Knapper^b

^a Centre for Food Innovation (Food Group), Sheffield Hallam University, Howard Street, Sheffield S1 1WB

^b Regional Food Group Yorkshire, Grimston Grange, 2 Grimston, Tadcaster, LS24 9BX

*Corresponding author email address: a.fairclough@shu.ac.uk

Abstract:

To our knowledge the cheese industry both Nationally and Internationally, is aware of the loss in colour of pre-packaged internally mould ripened blue cheeses (e.g. The American blue cheese AMABlu - Faribault Dairy Company, Inc.); however, after reviewing data published to date it suggests that no work has been undertaken to explain why this phenomenon is occurring which makes the work detailed in this paper novel. The amount and vivid colour of blue veins of internally mould ripened cheeses are desirable quality characteristics. It is therefore important that there is a sufficient amount of veining and that it maintains its blue appearance to be appealing to consumers therefore leading to maximised sales potential and profit for the manufacturing company.

The work undertaken in this study determined that the factors for optimum *in vitro* growth of *Penicillium roquefortii* (strain PRB6) were: a temperature of 20 °C ± 1 °C, pH of 6.0 ± 0.1, and a relative humidity of 70 %. Optimum *in vitro* growth mimicking the conditions typically found in pre-packed blue cheeses, and using

lactose as the sole carbon source, was facilitated by a gas mixture of 5 % Oxygen/0 % carbon dioxide/balance nitrogen).

Further *in vitro* studies have also shown that the increasing 'in pack' carbon dioxide concentration not only depresses the growth of *P roquefortii* but also affects immature conidiospore pigmentation (no effect has been seen on mature conidiospore pigmentation).

The implications of this study suggest that the majority of pre-packed internally mould ripened blue cheeses on sale in supermarkets are packaged in inappropriate materials. For some cheeses (e.g. the Roquefort-type cheeses) this is not an issue since these are packed in a much more mature state and some loss of veining colour is not appreciably noticeable; however, for less mature cheeses (i.e. those intended to continue maturing 'in-pack') any loss in colour has a significant impact on the cheese as well as on consumer perception.

Keywords: Consumer preference, Optimum growth conditions, Packaging conditions, *Penicillium roquefortii*, Pigmentation and colour, Pre-packed blue cheeses

Introduction:

The UK Cheese market was worth £2.1 billion in 2008 and increasing prices and modest growth in production volumes suggest that it will be worth £2.2 billion in 2009. Further growth is estimated at 4.5% for volume sales for the period 2009-2014 (Mintel 2009).

The use of the EU Protected Names Scheme to identify heritage and foods of cultural importance has helped consumers to identify products and their provenance. There are a total of thirty-seven protected foods in the UK; fourteen of these are

cheese of which five are blue veined cheeses, with Blue Stilton being the one that has the greatest renown. However, Buxton Blue, Dorset Blue, Exmoor Blue, and Dovedale are also recognised. Recent changes in the application process mean that more contemporary products such as Mrs. Bells Blue will become eligible to apply for the protection and enjoy the benefits in terms of increased consumer awareness and premium retail prices.

The filamentous fungus *Penicillium roquefortii* has been used extensively in the dairy industry to add flavour and veining to internally mould ripened blue cheeses (Engel and Teuber, 1989); its proteolytic and lipolytic enzymatic pathways have been well characterized biochemically (Genet *et al.* 1997). *P. roquefortii* produces two lipases extracellularly one with an acidic optimal pH and the other with an alkaline pH optimum (Mase *et al.* 1995, Lamberet and Menassa 1983, Menassa and Lamberet 1982, Lobyreva and Marchenko 1980, Eitenmiller *et al.* 1970). The relative importance of these lipases has not been fully elucidated. The acidic lipase has an optimal pH of approximately pH 6.5-6.8 which is very similar to that prevailing in the cheese (Lamberet and Menassa 1983); however, the alkaline lipase shows its most significant activity against butterfat (pH 8.0).

At the point of purchase, once price and taste has been considered, the consumer makes their decision on the visible characteristics of the product – they ‘eat with their eyes’ (Schröder 2003). Pre-packed cheeses typically have a viewing window for the consumer to assess the condition of the product. How does a consumer analyse the tastiness of the cheese from its appearance? For blue cheeses there must be a strong correlation between the quality and quantity of the veining and the strength of flavour. A large quantity of dark blue veining would be associated with a stronger flavour than one with a little light blue veining and a

consumer will make their choice based on personal preference for strength of flavour. If the veining has discoloured to green or brown there is a feeling that the cheese is over matured or even 'off' and as a result the decision is made not to purchase the cheese.

Traditionally, blue cheeses are retailed from delicatessen counters where the environmental conditions support the maintenance of the mould and the quality and characteristics of the product. Modern developments in food retailing mean that more food is sold in supermarkets and in pre-packed portions. The packaging creates an 'unnatural' condition for blue cheese to maintain its optimum quality as the living mould changes the atmosphere within the pack. If the product is not purchased while the atmosphere supports the maintenance of the mould, the mould will deteriorate from blue to green/brown. Further stress is placed on the mould through the distribution and retail chain: it can be subjected to fluctuating temperatures (despite the regulatory need to maintain a consistent temperature below 8 °C) and subjected to a shelf life of something like 56 days.

The packaging of internally mould ripened blue cheeses prior to the point of sale is controllable and an important contributing factor to maintaining the amount of blue veining making it visually appealing to the consumer. Other variables such as shelf life at point of purchase and temperature are beyond the control of the producer. This paper investigates the factors which contribute to this loss of colour/mould deterioration and how these may be overcome by understanding the optimum conditions for internally mould ripened blue cheese. By understanding these conditions, producers and retailers will be better able to develop packaging solutions that support and maintain the products' intrinsic and important characteristics and thereby maximise sales and minimise waste.

To our knowledge the cheese industry both Nationally and Internationally, is aware of the loss in colour of packaged internally mould ripened blue cheeses (e.g. The American blue cheese AMABlu - Faribault Dairy Company, Inc.). However, after reviewing data published to date, little or no work has been undertaken to explain why this phenomenon is occurring which makes the work detailed in this paper novel. The published literature shows there have been three studies undertaken by Golding in the 1930s and early 1940s on the gas requirements of *P. roquefortii*, and even in these studies Golding himself highlights flaws in some of the work undertaken. Golding subsequently undertook one study on the growth of *P. roquefortii* on synthetic media (Golding, 1934, 1937, 1940a, 1940b). Data obtained from these studies seem to have remained an academic exercise and, as such, have never been related to pre-packed internally mould ripened blue cheeses at the point of sale which makes this study novel. The aim of this study was to prove there is a link between pigment loss and packaging conditions.

Materials and Methods

In order to determine and understand the factors affecting the change in colour of the *P. roquefortii* within the cheese (*in-vivo*) it was first necessary to determine optimum *in-vitro* growth conditions.

For all the experimentation undertaken in this paper ASTM (American Society for Testing and Materials) Nutrient Salts Medium was used as a basal minimal medium supplemented with either glucose or lactose (to a final concentration of 1% w/v) as sole carbon source. This growth medium is an accepted mineral salt medium which will not support the growth of fungal cultures unless supplemented by the addition of a defined carbon source (ASTM Standards G-21, 1980). In our

experimental procedures glucose was used as the control carbon source for comparative purposes.

Maintenance of cultures

Stock cultures of *P. roquefortii* (strain PRB6) were maintained on potato dextrose agar (obtained from Oxoid Ltd.) slopes and stored at 4 °C.

This strain (PRB6) of *Penicillium roquefortii* is recommended for blue vein cheeses and will tolerate a low oxygen environment (5%).

Media used:

Basal ASTM Formulation (Table 1) - weights described below relate to the composition of medium per litre.

Table 1:

In-vitro determination of the optimum growth temperature.

Fresh cultures of *P roquefortii* were grown on potato dextrose agar (PDA) plates from which 4 mm plugs were cut and aseptically transferred to the centre of ASTM test plates, supplemented with either glucose or lactose (at a final concentration of 1% w/v). These plates were subsequently incubated at different temperatures ranging from 5 to 35 °C (± 1 °C) for 7 days and the amount of growth determined by measuring the average colony diameter (Figure 1a).

In-vitro determination of the optimum pH.

Basal ASTM medium was prepared and supplemented as previously described. However, since the medium was used to determine the pH optimum for *P. roquefortii* growth over a wide pH range (5.0 to 9.0) additional buffering was necessary, therefore organic (MES and Bicine) buffering salts were incorporated into the basal ASTM when the required pH fell outside the buffering range of the inorganic phosphates. The plates were incubated at the previously determined optimum temperature, and the amount of growth determined by measuring the average colony diameter (Figure 1b).

In-vitro determination of optimum relative humidity.

Freshly grown *P. roquefortii* (4 mm plugs) were aseptically placed onto the centre of ASTM plates (determined optimum pH) supplemented with either 1 % (w/v) of lactose or glucose and incubated for 7 days over a range of relative humidities (40 - 90 %) in a Modular Atmosphere Controlled (MACs) workstation. The amount of growth was determined by measuring the average colony diameter (Figure 2a).

In-vitro effects of atmosphere on conidiophore production and spore pigmentation:

P. roquefortii (strain PRB6) was grown in differing atmospheres of carbon dioxide ranging from 0 to 20 % (containing 5 % oxygen, balance nitrogen) on ASTM plates (optimally determined pH) supplemented with a sole carbon source as previously described. The plates were incubated for 7 days at the determined optimum relative humidity; and average colony diameter used as the assessment of the amount of growth (Figure 2b). The concentration of the individual gases reaching the mould cultures was accurately controlled (± 0.1 %) using the data access unit of the

Modular Atmosphere Controlled (MACs) workstation (Supplied by Don Whitley Scientific, Shipley, West Yorkshire, UK).

Results

It should be noted that the average growth data presented for the determination of each of the growth conditions is based on a data set of n=50 (to ensure reproducibility).

The atmosphere in which the *Penicillium roquefortii* is maturing post-packaging affects not only conidial colour but also the way in which conidiophores are produced and develop morphologically.

From the results obtained, the factors for the optimum *in vitro* growth conditions for *P. roquefortii* (strain PRB6), were: a temperature of 20 °C ± 1 °C, a pH of 6.0 ± 0.1, and a relative humidity of 70 %. Optimum *in vitro* growth using lactose as the sole carbon source was facilitated by a gas mixture of 5 % Oxygen/0 % carbon dioxide/balance nitrogen).

Further *in vitro* studies have also shown that the increasing 'in pack' carbon dioxide concentration not only depresses the growth of *P roquefortii* (Figure 2b) but also affected conidiospore pigmentation (Figures 3A - D).

Figures 1a and 1b:

Figures 2a and 2b:

The level of CO₂ in the atmosphere in which *Penicillium roquefortii* (strain PRB6) is growing appears to affect conidial pigmentation as well as the way in which the conidiophores are produced e.g. in an atmosphere of 5 % oxygen (commensurate with the atmosphere found in an internally mould ripened blue cheese) the conidiophores arise from hyphae that are growing on or just below the

surface of the medium thereby producing a uniform velvety texture (Figure 3a); whereas, when higher levels of CO₂ ($\geq 10\%$) are present a more lanose or cotton-woolly appearance dominates possibly due to the production of conidiophores arising from a mass of aerial hyphae (Figure 3b).

Figure 3:

The results shown in Figures 3a - e; are mirrored exactly in the control samples i.e. *P. roquefortii* grown on ASTM medium (supplemented with 1 % Glucose) and grown under the same atmospheric conditions (data not shown).

It was observed that once the plates were returned to a 'normal' atmosphere containing approximately 20 % O₂/0.04 % CO₂ pigmentation returns to the conidiospores after 15 to 20 minutes. Figures 3D and E show the plate (★) which had originally been grown in an atmosphere of 5 % O₂/20 % CO₂ (balance N₂) after being returned to a 'normal' atmosphere. It should be noted that pigmentation does not fully return to the more wispy parts of the colony under *in-vitro* conditions. The return of conidiospore pigmentation has also been observed when very lightly pigmented pre-packed cheese is opened and exposed to the air.

Light appears to play little or no part in the return of the pigmentation to the conidia since these plates were grown in a Modular Atmosphere Controlled (MACs) workstation subject to natural daylight - therefore, the loss and subsequent return of pigmentation is most likely to be under the control of other processes which may be either genetic, biochemical (e.g. secondary metabolites) or metabolic and from the lack of published data available it would appear these mechanisms have not been fully elucidated with regard to *Penicillium* species in general and *Penicillium roquefortii* in particular. If spore pigmentation is under metabolic control then published research mainly associated with *Aspergillus* species, in particular *A.*

nidulans (Brown and Salvo 1994) would suggest that despite them being chemically diverse, all secondary metabolites are produced by a few common biosynthetic pathways and often in combination with morphological development.

Conclusions:

This study suggests that the majority of pre-packed internally mould ripened blue cheeses are packaged in inappropriate materials. For some cheeses (e.g. the Roquefort-type) this is not an issue. However, for less mature cheeses loss in colour has a significant impact on quality and consumer perception.

From this work and other investigations currently being undertaken (data not shown) it shows that 'mature' conidiospore colour is unaffected by carbon dioxide concentration - this would indicate the pigment is 'fixed' in some way upon maturation. It is impossible to speculate at this stage whether the spore pigment of *Penicillium roquefortii* is associated with or 'bound' to the conidiospore coat or is internalised within the spore itself.

Understanding the growth requirements of *Penicillium roquefortii* enables food manufacturers to optimise the production process to achieve consistent and uniform veining within the cheese. This in turn leads to the development and optimisation of a packaging system through distribution and retailing that will preserve the quality of the product meeting the needs of the end consumer.

This kind of analysis is important for food manufacturers working with live cultures. Selection of the mould (or bacterial) strain at the development stage will determine the most appropriate one to use in the food manufacturing system and supply chain, reducing development costs, minimising production costs and optimising product performance.

Further, improved understanding of the growth requirements of *Penicillium roquefortii* where packaging systems can be optimised will allow better shelf-life and the ability of manufacturers to export their products to an increasing number of consumers around the world who are demanding new food experiences.

The UK government published its vision for food – Food 2030 – on 5th January 2010 (DEFRA 2010). Its focus is on producing more food sustainably to feed more people globally. It is structured around six core issues one of which is waste. The government programme WRAP runs the campaign ‘Love Food, Hate Waste’ which encourages consumers to reduce the 8.3 million tonnes of annual household food waste. This research potentially provides the basis of the development of a packaging system to protect and promote the quality of a food that is made from a valuable agricultural/dairy product (milk) to which a lot of value has been added (processing, maturing, distributing, retailing) and thus prevent food waste.

Acknowledgements:

The *Penicillium roquefortii* (strain PRB6) spore suspension for this work was kindly donated by Shepherds Purse Cheeses - North Yorkshire.

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