***Bullera crocea* and *Bullera armeniaca*, Two New Yeasts from Fruit and Vegetables**

By R. W. M. BUHAGIAR,¹ D. YARROW² AND J. A. BARNETT³*

¹Agricultural Research Council, Food Research Institute, Colney Lane, Norwich NR4 7UA, U.K.

²Yeast Division, Centraalbureau voor Schimmelcultures, Laboratorium voor Microbiologie, Technische Hogeschool Delft, Julianalaan 67a, 2628 BC Delft, The Netherlands

³School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

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Two new ballistosporeogenous yeasts, *Bullera crocea* and *Bullera armeniaca*, are described. *Bullera crocea* was isolated from strawberries, cauliflowers and watercress, and *B. armeniaca* from cauliflowers and cabbage. Unlike *B. alba*, *B. dendrophila*, *B. piricola* and *B. tsugae*, these new species could not utilize lactose; *B. crocea* was distinguishable from *B. armeniaca* by the ability of the former to utilize melibiose.

INTRODUCTION

Amongst yeasts isolated from soft fruit and vegetables between 1973 and 1975 were some with symmetrical ballistoconidia. They differed from existing species of *Bullera* and are here named *Bullera crocea* and *Bullera armeniaca*. Both species form stable, non-pigmented colonies and also colonies containing a majority of either large or small cells. Some colonies contained thick-walled resting cells which, in the small-celled generation of *B. armeniaca*, commonly germinated to form short septate filaments. This paper describes the two species.

METHODS

Isolation and maintenance of strains. Eleven strains of *B. crocea* were isolated from cauliflowers (*Brassica oleracea* var. *botrytis* cultivar Keverne) grown in Cornwall, two strains from strawberries (*Fragaria ananassa* cultivar Cambridge Favourite) grown in Norfolk and one strain from watercress (*Rorippa nasturtium-aquaticum*) grown in Surrey. Two strains of *B. armeniaca* were isolated from the same cauliflowers and one strain from cabbages (*Brassica oleracea* var. *capitata* cultivar Stark Winter) grown in Lincolnshire.

The yeasts from strawberries were isolated as described by Buhagiar & Barnett (1971) as modified by Buhagiar (1975). Samples of 100 g were macerated with 100 ml 0.1 M-KH₂PO₄ in an MSE Atomix Laboratory Blender for 90 s. Isolations were made from surface colonies on Difco Bacto YM agar (pH 6.0) containing antibiotics, CaCO₃ and thiamin (Buhagiar, 1975). The strains from cauliflowers and watercress were obtained from suspensions produced by macerating 5 g samples, each in 20 ml media of 0.1% (w/v) Difco Bacto peptone and 0.5% (w/v) NaCl (pH 7.0). The strain from cabbages was obtained from leaf discs, macerated in distilled water containing 1% (v/v) Tween 80. Each sample from cauliflower or cabbage was blended in a Coleworth Stomacher (A. J. Seward, London) for up to 2 min, and the isolations were made from surface colonies on YM agar (pH 3.4), incubated for 4 or 5 d at 20 °C.

The yeasts were maintained on Difco Bacto potato glucose agar at 20 °C and subcultured every 14 d.

Cultural and morphological tests. The cultural characteristics were studied, examining strains on malt extract agar (Barnett *et al.*, 1983) after 3 d at 25 °C and after one month at 15 °C and 25 °C; yeasts on potato glucose agar were examined after 7 and 14 d at 20 °C. The microscopical appearance was studied of cells grown on malt extract broth, on 2% Difco Bacto glucose/yeast extract/peptone water after 3 d incubation at 25 °C, and also during exponential growth at 20 °C in shaken cultures of chemically defined liquid medium (Barnett & Ingram, 1955) with 50 mM-D-glucose as sole carbon source. In addition, the formation of ballistoconidia and thick-walled resting cells were studied in cultures on potato glucose agar and Difco Bacto corn meal agar and on carrot plugs (van der Walt, 1970) incubated at 20 °C for up to one month.

Tests for germination of ballistoconidia were made with (i) 2% (w/v) aqueous Oxoid Ionagar no. 2, (a) without additions, (b) with 4 mM-potassium phosphate and adjusted to pH 6.5, (c) with vitamins as in morphology agar (Wickerham, 1951), (d) as (c), but with 7.5 mM-(NH₄)₂ SO₄ (van der Walt, 1970); (ii) slide cultures of malt extract (17° Balling) with 2% (w/v) Ionagar no. 2; (iii) potato glucose agar; and (iv) carrot plugs. Media (i) and (ii) were inoculated with ballistoconidia, from cultures on potato glucose agar, soaked in sterile distilled water for three weeks at 5 °C or 20 °C (Fischer & Holton, 1957). Incubations were at 20 °C and 25 °C; the cells were examined for up to 28 d for the formation of promycelia and sporidia. Cells on media (iii) and (iv) were incubated at 20 °C for up to 28 d and subjected to nuclear staining (Ganesan & Swaminathan, 1958) or 10% (v/v) Giemsa stain (Improved R66, G. T. Gurr, Searle Scientific Services, High Wycombe, Bucks., U. K.). Ballistoconidia were detected as described by do Carmo-Sousa & Phaff (1962) and also nuclear-stained. Tests for filamentous growth were made with slide cultures of corn meal and potato glucose agars (van der Walt 1970).

Searches for ascospores were made (van der Walt, 1970) for up to six weeks, after cultivation at 25 °C on carrot plugs, potato plugs, malt extract agar, acetate agar, Gorodkova agar, potato glucose agar and V8 agar.

Physiological tests. Aerobic growth tests were as described by Buhagiar & Barnett (1971), but the tubes of liquid media were rocked at 20 °C for one month. Tests for semi-anaerobic fermentation in Durham tubes were as described by van der Walt (1970), but with 0.1 M-D-glucose as sole carbon source. Tests for detecting the formation of extracellular amyloid material, arbutin hydrolysis, acid production and gelatin liquefaction were as described by van der Walt (1970). The reaction on litmus milk (Oxoid CM 45) was tested (Lodder & Kreger-van Rij, 1952) and the method of Markovetz & Kallio (1964) modified by Buhagiar (1975) was used to test for the utilization of *n*-decane and *n*-hexadecane. Cellulolytic activity was tested as described by Eggers & Pugh (1962), but with 5 g cellulose l⁻¹ (Whatman no. 1 filter paper, ball milled). Pectinolytic activity was tested with an overlay of sodium polypectate on agar plates (Paton, 1959; Buhagiar, 1979). DNA base composition of the cells was estimated by the method of Marmur (1961), modified by Barnes *et al.* (1977) and Buhagiar (1979), with the type culture of *Candida parapsilosis* (CBS 604), giving *T_m* 85.8 °C, as a standard.

The colour chart of Rayner (1970) was used to compare colours, and the morphological descriptions of the cells follow the criteria of Ainsworth (1971).

RESULTS

Bullera crocea Buhagiar sp. nov.

In liqore definito cum D-glucoso in augendo ad 20 °C cellulae ovatae ellipsoidales, 3 µm ad 5.5 µm × 3.0 µm ad 7.5 µm interdum (circa 1%) 5.5 µm ad 7.5 µm × 7.5 µm ad 12.5 µm, singulae binae aut in catenis brevibus. Cellulae cum gemmes ambio apicibus formatae sunt. Interdum in agar farinae *Solanum tuberosum* cum D-glucoso, cellulae cum ballistoconidiis turbinatae et congruens, singulae raro binae, 3.0 µm ad 5.0 µm × 3.0 µm ad 5.0 µm formatae sunt e sterigmatibus singularibus (raro duobus aut trinibus). Interdum cellulae sphaeroidales aut ellipsoidales fusi et granulosi cum muris crassis 2.5 µm ad 5.0 µm × 2.5 µm ad 5.0 µm (raro ad 9.0 µm × 12.5 µm) aut singulae aut in catenis brevibus formatae sunt. In agaro farinae *Zea mays* et in agaro farinae *Solanum tuberosum* cum D-glucoso pseudohyphae et hyphae verae non formantur. Ascospores non sunt. In liqore definito crescit cum raffinose, melezitose, sucrose, maltose, trehalose, methyl alpha-D-glucoside (lente), cellobiose, salicino, melibiose, D-glucose, D-galactose, L-rhamnose, D-ribose (interdum lente), D-xylose, D-arabinose (lente), L-arabinose, succinato, citrato, lactato (lente, interdum tarde), ribitolo (lente), D-glucitol (interdum lente), D-mannitol, galactitol, *myo*-inositol (variabile). Non crescit cum lactose, L-sorbose, erythritolo, amylo, aut inulino. Kalii nitrates non assimilatur. Vitaminarum addendum non necessarium est. Fermentatio nulla. In 30 °C crescit, sed non crescit in 37 °C. Materia amyloidea formatur. Arbutinum finditur sed non sebum bovillum, lactem, cellulose aut pectinum citri. Urea hydrolysat. Acidum non formatur. Non assimilantur *n*-decanum et *n*-hexadecanum. Gelatinum liquidum fit (exiguum). Typus: CBS 6714 in Collectione Centraalbureau voor Schimmelcultures, Delft, isolata ex *Fragaria ananassa*. [Etymology: *croceus*, Latin adjective, saffron-coloured, yellowish.]

Macroscopic appearance. After 3 d at 25 °C, or one month at 15 °C, streak cultures on YM agar or potato glucose agar were saffron to luteous, glossy or semi-glossy, smooth, soft, raised with entire borders. On malt agar, the cultures were luteous to ochraceous. After 7 d at 20 °C on YM agar or on potato glucose agar, the colonies were raised with entire borders and 1.5 mm in

diameter. After prolonged subculturing on potato glucose agar, a few colonies were white and remained white on subculturing.

Microscopic appearance (Fig. 1*a*). The oval or ellipsoidal vegetative cells usually formed two to four buds near the apices, sometimes on short, slender spicules. Most cells measured 3.0–5.5 µm × 3.0–7.5 µm, but <1% measured 5.5–7.5 µm × 7.5–12.5 µm. A few colonies on potato glucose agar contained >85% of the larger cells. These small and large cell generations remained stable at 20 °C on potato glucose agar.

Up to 10% of cells of both small and large cell generations were thick walled and contained brown pigment. These resting cells were spherical to ellipsoidal, hyaline or granular and measured 2.5–5.0 µm × 2.5–5.0 µm (rarely up to 9.0 µm × 12.5 µm). Ballistoconidia were formed on sterigmata on both vegetative and resting cells, up to three sterigmata per cell and, occasionally, two ballistospores on a single sterigma. After 3 d incubation at 20 °C, the ballistoconidia were discharged; they measured 3.0–5.0 µm × 3.0–5.0 µm and were turbinate, thin walled, translucent and sometimes vacuolate. The ballistoconidia formed up to four buds anywhere on the cell, often at the point of severance from the mother cell; many of these buds developed into thick-walled resting cells. Ballistoconidia from cultures of small or large cell generations produced cells of corresponding size. Occasionally, ballistoconidia remained attached to the mother cells, sometimes developing into thick-walled cells, 5.5–8.0 µm × 7.5–12.5 µm, or sometimes forming sterigmata and so a chain of up to three cells. A few of the resting cells germinated to form short filaments, occasionally branched, never with clamp connections.

There was little growth under the coverslips of slide cultures, filaments being absent or rudimentary, confined to branching chains of cells near the edge of the coverslip. No clamp connections, teliospores or ascospores were observed, with either separate strains or mixtures of strains; the thick-walled resting cells did not form promycelia or sporidia on germination. Nuclear staining showed that the resting cells, vegetative cells and ballistospores of both small and large cell generations were monokaryotic.

Semi-anaerobic fermentation tests. No gas was trapped in the Durham tubes.

Aerobic growth tests. The results are given in Table 1.

Table 1. *Results of physiological tests*

Substrate	<i>B. crocea</i>	<i>B. armeniaca</i>	Substrate	<i>B. crocea</i>	<i>B. armeniaca</i>
Raffinose	+	+	Erythritol	—	—
Melezitose	+	+	Ribitol	S	S
Sucrose	+	+	D-Glucitol	+ or S	+
Maltose	+	S	D-Mannitol	+	+
α,α-Trehalose	+	S	Galactitol	+	+
Methyl α-D-glucoside	S	S	myo-Inositol	V*	+ or S
Cellobiose	+	S	Starch	—	—
Salicin	S	S	Inulin	—	+ or S
Arbutin	+	+	KNO ₃	—	—
Melibiose	+	—	D-Glucose without vitamins	+ or S	—
Lactose	—	—	D-Glucose (50%, w/v)	—	—
D-Glucose	+	+	25 °C	+	+
D-Galactose	+	+	30 °C	—	—
L-Sorbose	—	—	Starch formation	+	+
L-Rhamnose	+	+ or S	n-Decane	—	—
D-Ribose	+ or S	S	n-Hexadecane	—	—
D-Xylose	+	+	Urea hydrolysis	+	+
D-Arabinose	S	S	Cellulose hydrolysis	—	—
L-Arabinose	+	S	Pectin hydrolysis	—	—
Succinate	+	+	Acid production	—	—
Citrate	+	+	Litmus milk and fat test	—	—
Lactate	S or L	S or L	Gelatin liquefaction	weak	weak
Glycerol	S or L	S			

Explanation of symbols. Within 7 d of inoculation, growth in mg dry wt ml⁻¹, + = >0.4, — = <0.4; S = 0.05 to 0.2 mg dry wt ml⁻¹ within 28 d; L = growth only after 21 d; V = results varied between strains.

* Of 13 strains tested, 6 were —, 7 were S or L.

DNA base ratio. The mean of three determinations was 53.0 mol % G + C (T_m 91.1 $\pm$ 0.1 °C) for CBS 6714 and 52.6 mol % G + C (T_m 90.9 $\pm$ 0.1 °C) for two other strains.

The type strain of *Bullera crocea* is CBS 6714, isolated from strawberries, and is deposited at the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, the Netherlands.

Bullera armeniaca Buhagiar sp. nov.

In liquore definito cum D-glucoso in augendo ad 20 °C cellulae ovatae vel oblongae, 2.0 μ m ad 5.0 μ m $\times$ 2.5 μ m ad 7.5 μ m raro oblongae vel cylindricae interdum apiculatae, 3.0 μ m ad 5.0 μ m $\times$ 7.5 μ m ad 14.0 μ m (raro ad 24.5 μ m) singulae, binae aut in catenis brevibus. Cellulae cum gemmis singularibus aut duobus quae in aut juxta apice vel ambio apicibus formatae sunt. In agaro farinae *Solanum tuberosum* cum D-glucoso, interdum cellulae 5.0 μ m ad 10.5 μ m $\times$ 6.0 μ m ad 12.5 μ m, sphaeroidales, ellipsoidales, reniformae, oblongae aut turbinatae, fusci et granulosi, cum muris crassis aut singulae aut in catenis brevibus formatae sunt; e his cellulis, racemis parvis, singulae aut binae (raro ternae) et interdum bifurcae formantur. Cellulae cum ballistoconidiis turbinatae et congruens, 5.0 μ m ad 7.5 μ m $\times$ 5.0 μ m ad 7.5 μ m singulae raro binae formatae sunt e sterigmatibus singularibus (raro duobus aut trinibus). In agaro farinae *Zea mays* et in agaro farinae *Solanum tuberosum* cum D-glucoso, pseudohyphae et hyphae verae non formantur. Ascosporae non sunt. In liquore definito crescit cum raffinoso, melezitoso, sucroso, maltoso (lente), trehaloso (lente), methyl alpha-D-glucosido (lente), cellobioso (lente), salicino (lente), D-glucoso, D-galactoso, L-rhamnoso (interdum lente), D-riboso (lente), D-xyloso, D-arabinoso (lente), L-arabinoso (lente), succinato, citrato, lactato (lente, interdum tarde), glycerolo (lente), ribitolo (lente), D-glucitolo, D-mannitolo, galactitolo, *myo*-inositolo (interdum lente), inulino, (interdum lente). Non crescit cum melibioso, lactoso, L-sorboso, erythritolo aut amylo. Kalii nitras non assimilatur. Vitaminarum addendum necessarium est. Fermentatio nulla. In 30 °C crescit, sed non crescit in 37 °C. Materia amyloidea formatur. Non assimilantur *n*-decanum et *n*-hexadecanum. Urea hydrolysatur. Arbutinum finditur sed non sebum bovillum, lactem cellulosum aut pectinum citri. Acidum non formatur. Gelatinum liquidum fit (exiguum). Typus: CBS 7091 in Collectione Centraalbureau voor Schimmelcultures, Delft, isolata ex *Brassica oleracea* var. *capitata*. [Etymology: *armeniacus*, Latin adjective, apricot-coloured.]

Macroscopic appearance. After 3 d at 25 °C or one month at 15 °C, streak cultures on YM agar, potato glucose agar or malt agar were orange to apricot, glossy or semi-glossy, smooth, soft and with entire borders. After a week at 20 °C on YM agar or potato glucose agar, ~80% of the colonies were 2.0–3.0 mm in diameter and <20% were 0.05–1.0 mm in diameter. Some colonies were white, and subsequent cultures derived from these remained white.

Microscopic appearance. (Fig. 1b). The vegetative cells were oval to oblong, 2.0–5.0 μ m $\times$ 2.5–7.5 μ m, but a few were 3.0–5.0 μ m $\times$ 7.5–14.0 μ m (rarely up to 24.5 μ m). Commonly, one or two buds formed on or near either apex. The large and small colonies each contained large and small cells, <1% of the larger colonies containing >70% large cells.

Most colonies formed by small cells on potato glucose or maize meal agar, incubated for 5 d at 20 °C, formed thick-walled resting cells, 5.0–10.5 μ m $\times$ 6.0–12.5 μ m, often containing brown pigment. These cells were hyaline or granular, globose, ellipsoidal, kidney shaped, turbinate or oblong, and sometimes centrally constricted and septate. Such resting cells occurred only rarely in colonies of the larger cells. On carrot plugs, however, both cell types formed resting cells.

On potato glucose agar or carrot plugs, incubated for up to one month at 20 °C, the resting cells germinated to form short filaments, occasionally branched, without clamp connections and rarely with buds. These filaments were either short, undulating, often septate, hyaline mycelial hyphae, or short, distorted pseudohyphae, with up to five segments, budding on a wide base. The distal cell was often large, granular, curved or cymbiform, occasionally with an apical bud. The resting cell and the older segments progressively degenerated, leaving only the distal cell intact, and this readily became detached from the degenerated structure.

On potato glucose agar or maize meal agar, ballistoconidia were formed on sterigmata from resting cells and, occasionally, from vegetative cells. Many resting cells formed from one to three acicular to ventricose sterigmata, occasionally septate and up to 3 μ m long, at the apex of which one or two ballistoconidia were produced. Some ballistoconidia remained attached to the

Fig. 1. Cells of (a) *Bullera crocea* and (b) *Bullera armeniaca*. The yeasts were grown at 18 °C in shaken culture of Difco Bacto yeast nitrogen base with 25 mM-D-glucose (a) for 2 d to $A_{640} = 0.18$, (b) for 1 d to $A_{640} = 0.3$. They were photographed by L. K. Barnett with a Mark I Zeiss photomicroscope, using Nomarski optics and a planapochromatic objective, magnification $\times 100$, numerical aperture 1.32 (see Barnett *et al.*, 1983). The bar markers represent 10 μm .

mother cell and either formed further ballistoconidia on sterigmata or developed into intercalary or terminal thick-walled resting cells. A few ballistoconidia were formed on sterigmata on the filamentous structures which grew out of resting cells. The ballistoconidia were discharged after 6 d at 20 °C on maize meal agar; they measured 5.0–7.5 $\mu\text{m} \times 5.0$ –7.5 μm , and were turbinate, thin walled, translucent and sometimes vacuolate. Ballistoconidia from the small or large cell generations, discharged on to maize meal agar, give rise to colonies of cells of their respective sizes. These ballistoconidia formed up to four buds anywhere on the cell surface, or sometimes developed into thick-walled resting cells which, in turn, formed either sterigmata with ballistoconidia or filamentous structures.

Little growth occurred under coverslips on slide cultures with potato glucose agar or maize meal agar. Hyphae and pseudohyphae were absent. No clamp connections, teliospores or ascospores occurred in individual strains or mixtures of strains and the resting cells did not form promycelia or sporidia. Nuclear staining showed the cells to be monokaryotic.

Semi-anaerobic fermentation tests. No gas was trapped in the Durham tubes.

Aerobic growth tests. The results are given in Table 1.

DNA base ratio. The mean of three determinations was 55.5 mol % G + C (T_m 92.1 $\pm$ 0.1 °C) for CBS 6574 and 56.3 mol % G + C (T_m 92.5 $\pm$ 0.1 °C) for another strain.

The type strain of *Bullera armeniaca* is CBS 7091, isolated from cabbage, and is deposited at the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, the Netherlands.

DISCUSSION

Imperfect yeasts which form ballistoconidia are classified either in the genus *Bullera* Derx or in *Sporobolomyces* Kluyver et van Niel. Traditionally, taxonomists have classified in *Bullera* species with ballistoconidia which are symmetrical about their long axis, are non-pigmented and non-filamentous. Those with asymmetrical ballistoconidia, usually with pink or red pigments, and which form filaments have been classed as *Sporobolomyces* (Lodder & Kreger-van Rij, 1952; Lodder, 1970).

Table 2. *Differences between Bullera species*Results from Barnett *et al.* (1983). +, Growth within 7 d; D, growth in 7 to 21 d; —, no growth.

Growth test	<i>Bullera</i> species...	<i>alba</i>	<i>armeniaca</i>	<i>crocea</i>	<i>dendrophila</i>	<i>piricola</i>	<i>tsugae</i>
KNO ₃		—	—	—	—	+	+
Lactose		+	—	—	+	+	+
Melibiose		+	—	+	—	+	—
Raffinose		+	+	+	—	+	—
Galactitol		— or D	+	+	+	D	—
myo-Inositol		+	D	—	D	+	—
Without biotin		+	—	+	+	—	+
Mol % G + C		54.5*	55.9	52.7	.	.	.

* Nakase & Komagata (1971).

Stadelmann (1975) isolated a yeast from the leaves of pear trees, which he described as forming both symmetrical and asymmetrical ballistoconidia. He placed it in *Bullera*, altering the description of the genus to allow the inclusion of yeasts with asymmetrical ballistoconidia and with filaments, but not yeasts which are pink or red.

From the way *Bullera* and *Sporobolomyces* are now defined, there seems to be no essential and consistent difference between them; however, species with symmetrical ballistoconidia are excluded from *Sporobolomyces* and, up to now, those with reddish pigments from *Bullera*. Although the two species described here have symmetrical ballistoconidia, and therefore should not be classed as *Sporobolomyces*, both should also be excluded from *Bullera* because they are pigmented. However, as both species sport stable, unpigmented colonies, it seems admissible to place them in *Bullera*, rather than to exclude them merely because some colonies are pigmented.

Differences between the two species are summarized in Table 2. Although they are similar culturally, morphologically and in their physiological characteristics and habitat, they differ by 3.2% in the average percentage of G + C of the DNA. According to Phaff (1977), a difference of from 1 to 2% in DNA base composition is taxonomically significant. Indeed, Meyer *et al.* (1978) reported ranges differing by 0.7 to 2.2% (average 1.2%) in six species of *Hanseniaspora*; and Yarrow & Nakase (1975) found a difference of 2.5% between the highest and lowest values for strains of *Saccharomyces cerevisiae*. Hence, the finding of a difference of 3.2% for the two groups of yeasts described above justifies placing them in two taxa.

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