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Metabolism of volatile phenolic compounds from hydroxycinnamic acids by *Brettanomyces* yeast

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Abstract. The formation of 4-ethyl and 4-vinyl derivatives of guaiacol, phenol and syringol from ferulic acid, *p*-coumaric acid and sinapic acid, respectively, by *Brettanomyces* sp. in a synthetic medium was studied by gas chromatography-mass spectrometry. Some of these metabolites possess strong spicy, smoke-like, medicinal, clove-like, woody or phenolic odours and their role as spoilage compounds in wine is discussed. Their formation appears to be characteristic of this yeast genus and its sporulating form *Dekkera*, suggesting these yeasts are Pof^+. This paper attempts to clarify the distinctive and 'characteristic' odours which have long been attributed to *Brettanomyces* yeast metabolism.

Key words: *Brettanomyces intermedius* – *B. anomalus* – Wine spoilage – Ferulic acid metabolism – *p*-Coumaric acid – Sinapic acid – 4-Ethyl phenol/guaiacol/syringol – 4-Vinyl phenol/guaiacol/syringol – Spicy/clove-like odour

Brettanomyces has long been recognised as a spoilage yeast in fermented beverage production, imparting undesirable odours and flavours to wine, beer and cider. The production of these off-odours has been recognised as a characteristic of *Brettanomyces* species and has been included as a criterium for the characterisation of this genus (van der Walt 1961; Gilliland 1962; Lodder 1970). Peynaud and Domercq (1956) first distinguished and described these odours. Amongst aroma notes such as sour, sharp and disagreeable, they distinguished a fruity, aldehydic odour similar to that of apples. They also reported an odour consistently present which they described as 'mouse-like'. They attributed this aroma to acetamide, however, it has since been reported that 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-3,4,5,6-tetrahydropyridine are responsible for the strong mousy flavour and aroma produced by *Brettanomyces* (Strauss and Heresztyn 1984). Van Zyl (1962) also noted that an apple-like aroma arose when grape must was fermented by *Brettanomyces*, and became more noticeable toward the end of fermentation. In addition, Van Zyl et al. (1963) recognised a peculiar and characteristic flavour in grape juice fermented by *Brettanomyces* and attributed its presence to two unidentified substances which they considered characteristic of this yeast's metabolism.

During investigations into the nature and cause of the mousy flavour in Australian wine (Heresztyn 1986), a distinct aroma described variously as cider-like, spicy, clove-like or phenolic, developed in grape juice and malt extract medium fermented by five *Brettanomyces* species. This aroma, as well as a 'mouse-like' odour and flavour, were formed toward the end of fermentation. The production of phenolic compounds by yeast, bacteria and moulds from substituted cinnamic acids has been well documented (Steinke and Paulson 1964; Maga 1978; Dubois 1983). Goodey and Tubb (1982) carried out genetic and biochemical analysis of strains of *Saccharomyces* yeast capable of producing a phenolic off-flavour and assigned them the phenotype Pof^+. Despite considerable investigation into the off-odours and flavours produced by *Brettanomyces*, the compounds responsible for the spicy/phenolic odour produced by this yeast have apparently not been identified.

This paper reports the formation and identity of volatile phenolic compounds by *Brettanomyces* yeast. The metabolism of these compounds from hydroxycinnamic acids is demonstrated and their effect on the sensory quality of wine is discussed.

Materials and methods

Microorganisms. Grape juice fermentations were conducted by *Brettanomyces anomalus* (CBS 77) and *Brettanomyces intermedius* (CBS 73), obtained from the Yeast Culture Collection at the Centraalbureau voor Schimmelcultures, Delft. *Saccharomyces cerevisiae* strain No. 70 from the Institute's Collection was chosen as a representative wine yeast. A strain of *B. intermedius* (BW-1), isolated from a tainted wine submitted to the Institute by a winery, was used in fermentations of chemically defined media.

Media and fermentation conditions. 25 mg each of ferulic acid, *p*-coumaric acid, caffeic acid, sinapic acid, syringic acid and vanillic acid were individually added to 150 ml of chemically defined medium of the following composition: glucose 50 g; $(NH_4)_2SO_4$ 2 g; KH_2PO_4 2 g; $MgSO_4 \cdot 7\,H_2O$ 0.25 g; $CaCl_2$ 0.25 g; $ZnSO_4 \cdot 7\,H_2O$ 2 mg; $FeSO_4 \cdot 7\,H_2O$ 10 mg; citric acid 0.2 g; biotin 30 µg; thiamine 1 mg; pyridoxine 1 mg; nicotinic acid 10 mg, made up to 1 l with distilled water. Following inoculation with approximately 10^6 cells/ml of *B. intermedius* (BW-1), the ferments were incubated at 25°C for 3 weeks after which they were centrifuged at 10,000 rev/min for 20 min. The supernatants were stored at −4°C prior to analysis for phenolic compounds. Two white wines prepared by fermentation of grape juice by *B. intermedius* (CBS 73) and *B. anomalus* (CBS 77) developed strong spicy, phenolic odours toward the end of fermentation and were also stored for future

analysis. A white wine fermented from the same grape juice with *Saccharomyces cerevisiae* was analysed as a control.

Extraction procedure for phenolic compounds for GC-MS analysis. The ferments were acidified to pH 2 with 10% HCl prior to continuous extraction with Freon F11 for 24–30 h. The organic phase (about 250 ml) was then extracted in a separatory funnel with 1×80 ml, 2×50 ml of pH 8.5 $NaHCO_3$ solution, followed by extraction in the same manner with 0.5 M NaOH. This second alkaline fraction containing the phenolic compounds, was adjusted to pH 2 with conc. H_2SO_4 and extracted with 3×80 ml of diethyl ether. After washing with 2×50 ml of distilled H_2O, the ether extract was dried over $MgSO_4 \cdot 3H_2O$ for 30 min, filtered, distilled on a water bath through a column of Fenske's helices to a final volume of approximately 100 µl and stored at −4° C.

Gas chromatographic and mass spectrometric analysis. GC-MS was carried out using a Finnigan 4021 GC-MS Data System, equipped with a 25 m quartz silica BPl column (SGE, Melbourne, Australia). The oven temperature conditions were 80° C for 2 min, then programmed at 1° C/min to 100° C, at which point the rate of heating was changed to 4° C/min until a final temperature of 220° C was reached.

Identification of compounds was made by comparison with authentic reference samples or with published mass spectra.

Gas chromatography with sniff detection. Gas chromatography combined with effluent sniffing (Dravnicks and O'Donnell 1971) was carried out on a modified Varian Aerograph 1400 GC fitted with a Carbowax 20M glass SCOT column. The oven temperature was held at 60° C for 10 min then programmed at 1° C/min to 180° C.

Results and discussion

Two white wines produced by fermentation with *Brettanomyces anomalus* and *B. intermedius* both contained large quantities of 4-ethyl phenol and 4-ethyl guaiacol as well as a trace amount of 4-vinyl guaiacol. These compounds were identified by comparison with authentic reference samples. No phenolic compounds were detected in the *Saccharomyces* ferment. The aroma of the *Brettanomyces* wines was strongly spicy and clove-like. A strong mousy character was also apparent (Strauss and Heresztyn 1984). GC effluent sniffing of phenolic fractions of these two wines revealed two major areas of interest, one of which was strongly spicy and clove-like in character, and the other spicy but more smoky. These two regions on the chromatogram corresponded to retention times of authentic samples of 4-ethyl guaiacol and 4-ethyl phenol, respectively, chromatographed under the same GC conditions with most of the column effluent directed to the flame ionisation detector. Regions of the chromatogram where the spicy odour was fading, at times resembled an apple or cider-like character.

Experiments with a synthetic medium were then undertaken to determine which hydroxycinnamic acids *Brettanomyces intermedius* (BW-1) could metabolize to produce the phenolic compounds found in these wines. Under the conditions described, this yeast metabolized ferulic, *p*-coumaric and sinapic acids, but appeared unable to utilize caffeic, vanillic or syringic acids. Table 1 lists the phenolic products from the above three hydroxycinnamic acids, in their relative quantities as determined by GC-MS.

Table 1. Relative abundance of metabolic products formed from hydroxycinnamic acids by *Brettanomyces* yeast in chemically defined media

Compound	Substrate		
	Ferulic acid	*p*-Coumaric acid	Sinapic acid
Phenol*		trace	trace
4-Ethyl phenol*		+++	+
4-Vinyl phenol		trace	
4-Ethyl guaiacol*	+++		
4-Vinyl guaiacol*	trace		
Unknown	+		
4-Ethyl syringol			+++
4-Vinyl syringol			++

Compounds marked with an asterisk were identified with an authentic reference sample. Tentative identification of the other three phenols was made by comparison with published mass spectral data (Tressl et al. 1976)

The ferulic acid ferment possessed a strong smoky, clove-like aroma which was attributed to the large amount of 4-ethyl guaiacol formed. This compound has been described as possessing smoky, clove-like, spicy odour notes, depending on its concentration (Dubois 1983). Furthermore, GC-effluent sniffing of this phenolic extract showed a strong spicy, clove-like region in the chromatogram at the same retention time of authentic 4-ethyl guaiacol. An unknown compound produced during this fermentation had the following mass spectrum: m/z (relative intensity); 182(34), 167(1), 165(1), 153(4), 137(100), 125(9), 122(17), 110(4), 106(9), 93(27), 77(8), 65(24). The apparent mol.wt. of 182 and the similarity of the spectrum to that of compounds such as dihydroconiferyl alcohol, and homovanillic acid, a component of Japanese wine (Shimizu and Watanabe 1982), suggests this unknown may be an intermediate in ferulic acid metabolism. Enoki et al. (1981) and Gupta et al. (1981) discuss reductive and oxidative transformations of ferulic acid to a wide range of cinnamic acid derivatives by lignin-degrading white-rot fungi. The formation of this unknown by *Brettanomyces* may be related to one of these pathways.

A powerful woody, smoky aroma was evident toward the end of fermentation of the chemically defined medium to which *p*-coumaric acid had been added, and remained noticeable during the phenolic extraction procedure. 4-Ethyl phenol, which has a strong woody, phenolic odour (Maga 1978; Dubois 1983) was identified as the major volatile component, suggesting this compound contributes to the overall phenolic aroma of wines infected by *Brettanomyces*. This was confirmed by GC-effluent sniffing. 4-Vinyl phenol possesses a strong smoky or medicinal odour even at very low concentration and may also have contributed to the aroma of this ferment to some extent.

Sinapic acid was the only other phenolic acid examined which the yeast appeared able to metabolize. Dubois (1983) reported the syringols or dimethoxyphenols to have weak

odours, and indeed, the experimental ferment did not have a distinguishable phenolic aroma.

The formation of 4-ethyl guaiacol, 4-ethyl phenol and their vinyl analogues in both grape juice and chemically defined ferments by *Brettanomyces* yeast demonstrates this organism's ability to produce volatile phenols from ferulic and *p*-coumaric acids. The yeasts examined here also have the ability to metabolize sinapic acid in the same way, under the conditions described. Thermal fragmentation of these three compounds in model reactions yielded the phenolic products identified in these *Brettanomyces* fermentations as well as other components (Tressl et al. 1976). Yeast of other genera including certain *Saccharomyces* strains, as well as bacteria, are also known to metabolize some of these compounds in beer and wine and are generally considered to be spoilage organisms (Steinke and Paulson 1964; Maga 1978; Goodey and Tubb 1982). Goodey and Tubb (1982) did, however, examine wine and distilling strains of *Saccharomyces* that were Pof^+. Pof^+ yeasts were able to decarboxylate coumaric and cinnamic acid as well as ferulic acid, which they attributed to a nuclear gene POF1. The *Brettanomyces* yeasts examined here appear to be Pof^+, and the *Saccharomyces* strain chosen, Pof^-. The decarboxylation of sinapic acid by these yeasts may also be coded for by the POF gene.

Formation of ethyl analogues of the phenols was not discussed by Goodey and Tubb (1982), however, in their work on steam-volatile phenols, Steinke and Paulson (1964) hypothesized that first cinnamic acids are decarboxylated to vinyl phenols, followed by reduction of the vinyl group to yield the ethyl analogue.

The phenolic acids discussed here are natural components of grapes and wine, malt and beer. They may be present in the free form, or as esters or glycosides (Maga 1978; Dubois 1983). The ability of certain yeast and bacteria to metabolize these compounds has important consequences to fermented beverages. Volatile phenols can be considered as natural components in wines and beer, or as spoilage compounds when present in excessive amounts. The ability of *Brettanomyces* yeast to produce volatile phenols appears to be an important feature of this yeast's metabolism, and the work reported here suggests these phenols are major contributors to the distinctive and characteristic odours and flavours, other than mousiness, produced by this yeast. Unpublished results indicate *Dekkera*, the sporulating form of this yeast, also has the ability to produce spicy, phenolic odours as well as a mousy taint upon growth in nutrient broth or grape juice.

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