

**THE USE OF YEAST INOCULATION IN FERMENTATION FOR PORT
PRODUCTION; EFFECT ON TOTAL POTENTIAL ETHYL CARBAMATE.**

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SUMMARY

A commercial wine yeast *Saccharomyces cerevisiae* UCD 522 (pre-cultured in the presence of certain mass-labelled amino acids) was inoculated into a port must which was then allowed to ferment under controlled conditions of temperature and agitation. The influence of potential ethyl carbamate (EC) precursor formed due to yeast pre-culture, upon total potential EC levels was studied at various stages of fermentation. Pre-culture accumulation did not give rise to detectable levels of EC precursor during port fermentation.

INTRODUCTION

The work presented here represents a study of the effect of inoculation with a commercial preparation of *Saccharomyces cerevisiae* on total potential ethyl carbamate (EC) levels in finished ports. The production of EC and its precursors (compounds which can react chemically to give EC) in fermented beverages and their derivatives, particularly due to the metabolism of arginine by *S. cerevisiae*, has been the subject of considerable study. Concern about this compound is related to its status as a known animal carcinogen (Pound, 1972). There is some dispute as to whether toxicity data may be extrapolated to demonstrate a risk to man (Schlatter & Lutz, 1990) however, concern has been aroused by the the Canadian government's imposition, in 1985, of legal limits for EC in imported beverages. There is general agreement that all efforts must be made to ensure that levels of this compound are maintained as low as possible in foods and beverages. Port, from the Douro region of northern Portugal, is traditionally vinified without the addition of any exogenous yeast, however the use of commercial yeast preparations is becoming more frequent. Of specific interest in here is that EC arising from precursor compounds which have accumulated within the yeast prior to must inoculation (i.e. in the preparation of the inoculum), either that released into the must during fermentation, or that liberated from the yeast following fortification. The methodology employs laboratory scale micro-vinifications inoculating with a strain of *S. cerevisiae* pre-cultured in the presence of mass-labelled amino acids (¹⁵N aspartic acid and ¹³C arginine) as predominant nitrogen sources. Control pre-cultures were prepared in the same way using non-labelled amino acids. Samples were withdrawn from the fermenting must at specific intervals and label incorporation into specific compounds was followed using high resolution mass spectroscopy.

MATERIALS AND METHODS

Reagents: All reagents were GPR unless otherwise stated. Labelled ¹⁵N glutamic and aspartic acids were obtained from Sigma. Labelled arginine compounds (L-arginine-7-¹³C or guanidino ¹³C arginine) were obtained from K&K Greeff Ltd. Croydon, Surrey.

Preparation of yeast inocula for port fermentations: *S.cerevisiae* UCD 522 (Uvaferm CM, Danstar) was initially propagated from the commercial preparation by plating on WL Nutrient Medium (Difco). Six well isolated, typical colonies were taken, washed twice and finally suspended in 10 ml sterile deionised water giving a total cell count of approx 6.0×10^7 cell/ml. Yeast Carbon Base (YCB [Difco]), prepared according to the manufacturers instructions, was sterilised by membrane filtration and stored in aliquots of 25ml. Media A, B, C & D were prepared in this YCB according to Table 1, corresponding cultures being prepared by the addition of 50 μ l of the yeast suspension described above. The initial culture density being 1.2×10^5 cells/ml which, following incubation (30°C, 48h, 100rpm) reached 2.0×10^8 cells /ml.

Table 1 Nitrogen sources in pre-culture media (mg in 25ml total volume*)

Medium	Arginine	¹³ C Arginine	Aspartic acid	¹⁵ N Aspartic acid	Glutamic acid	GABA
A	-	50.0	2.5	-	2.5	50.0
B	50.0	-	2.5	-	2.5	50.0
C	-	-	-	30.0	30.0	50.0
D	-	-	30.0	-	30.0	50.0

*All compounds were prepared as concentrated solutions and autoclaved separately.

Prior to inoculation into must, cells were harvested and washed 3 times with appropriate isotonic wash solutions; in an attempt to minimise osmotic effects on the "leakyness" of cells prior to inoculation,. After the 3rd washing the cell suspension was harvested and resuspended in a 0.5ml of wash solution prior to inoculation into must.

Must fermentations and sampling

Thermovinified must was employed as the fermentation medium; this was prepared during the 1993 vintage by heating unfermented red must to approximately 70°C for 20 minutes, the resulting must (hexose sugar content of 210 g/l) was stored at -8°C until use. Four fermentations, referred to as A,B,C&D, corresponding to the media used in the preparation of the inocula (Table 1) as 200ml in 500ml conical flasks (30°C and 100rpm, in duplicate) were carried out in thermovinified must. Samples were withdrawn from all 4 fermentations when the sugar content had reduced to 185 g/l these samples were fortified to 20 % v/v ethanol, fortification taking place both in the presence (on) and absence (off) of suspended yeast. All fermentations were then allowed to continue fermenting until sugar content had reduced to ca. 130 g/l (i.e. the usual point at which port fermentations are arrested) at which point the cells from fermentations A,B&C were removed by centrifugation, the musts being retained. Cells from fermentation A were washed with an appropriate isotonic wash solution, resuspended in 0.5 ml of wash solution and then introduced into the retained must of B. In the same way cells from must B were introduced into the retained must C (the possibility of discriminating between ¹³C and ¹⁵N labelled ethyl carbamate species enabling the retained musts to be used as control musts for the other label). Samples were then withdrawn from A,B and C and fortified on and off the yeast (as above). Fermentations were then allowed to continue until 20mg of ¹⁵N aspartic acid were added to fermentation C with approximately 10g/l of fermentable sugars remaining. After fermentation to dryness samples were withdrawn from fermentations C and D and fortified on and off the yeast (as above). It should be noted that port producers, in addition to the classical fortification timing, often make quantities of both very sweet (geropiga) and dry wines for blending purposes. All samples thus obtained were heated for 48hrs. at 80°C to encourage EC production form precursors (Monteiro *et al* 1989) prior to analysis for both EC and mass labelled ECs.

EC analyses

Extraction: All samples were extracted according to the method of Dennis *et al* (1986). Concentration was improved using the contents of 3 sachets of Extrelut column refill material (BDH) for each analysis and extracting 60 ml of diluted sample (1 in 4). Elution from the column was with dichloromethane and concentration by evaporation at 55 °C using Vigreux distillation columns. Final clean-up was achieved via a Florisil Sep-Pak (Phase Separations), EC being eluted with 7% methanol in dichloromethane.

GCMS: EC was separated prior to mass spectral analysis using a Carlo Erba Mega Series GC. A Superox II column (Alltech) (L=50m, 0.32mm diam, 0.3µm film thickness) was used with direct on-column injection and a temperature programme of 60°C for 1min, 8°C/min to 120°C, 15°C/min. to 200°C, hold at 200°C for 5 min. Under these conditions EC eluted in 14.5 mins. The GC was connected to a Kratos MS80RFA mass spectrometer analysis for EC being carried out in SIM. mode. Nominal instrument resolution was 3000 for monitoring unlabelled EC fragment ions (m/z 62.0242) and 10000 for monitoring ¹⁵N and ¹³C fragments (m/z 63.0213 and m/z 63.0276 respectively), start mass was m/z 54.0469, lock mass m/z 57.0704. Dodecane was used as the calibration standard.

RESULTS AND DISCUSSION

Table 2 shows concentrations of total and mass-labelled EC derived from integrated peak areas from GCMS chromatograms - all samples having been heated for 48 hrs. at 80°C

Table 2. Ethyl carbamate analyses (ng g⁻¹)

Sample:	Total EC	¹² C EC	¹³ C EC (% tot)	¹⁵ N EC (% tot).
Sampling 1 (Fortification when 185 g L ⁻¹ hexose sugars remain)				
Culture A on yeast	137	135	1.18 (0.86)	0.53 (0.39)
Culture A off yeast	52	51	0.44 (0.85)	0.16 (0.31)
Culture B on yeast	127	125.	1.37 (1.08)	0.44 (0.35)
Culture B off yeast	76	75	0.73 (0.96)	0.28 (0.37)
Culture C on yeast	159	156	2.02 (1.27)	0.67 (0.42)
Culture C off yeast	66	65	0.71 (1.07)	0.29 (0.44)
Culture D on yeast	179	177	1.65 (0.92)	0.84 (0.87)
Culture D off yeast	92	91	1.10 (1.14)	0.41 (0.44)
Sampling 2 (135 g L ⁻¹ hexose sugars remaining) cells in ¹³ C-free must.				
A cells - on yeast	71	70	0.68 (0.96)	0.21 (0.29)
A cells - off yeast	54	53	0.44 (0.81)	0.22 (0.40)
B cells - on yeast	94	93	0.94 (1.00)	0.39 (0.41)
B cells - off yeast	70	69	0.62 (0.89)	0.34 (0.49)
Sampling 3 (fermented to dryness)				
Culture C*- on yeast	184	181	1.69 (0.92)	1.32 (0.72)
Culture C* - off yeast	124	122	1.23 (0.99)	1.00 (0.81)
Culture D** - on yeast	80	79	0.94 (1.18)	0.31 (0.39)
Culture D** - off yeast	46	45	0.47 (1.03)	0.21 (0.46)

* after addition of ¹⁵N labelled aspartic acid when 10 g l⁻¹ remain.

** contol - no addition of arginine.

In every case samples fortified in the absence of yeast contain lower levels of potential EC than the corresponding samples fortified prior to yeast removal, suggesting that fortification liberates intracellular precursors. Results from samplings 1 and 2 suggest that, whilst the largest amount of EC precursor is present at a point where fermentation has just started, there appears to be no significant contribution to this total attributable to yeast excretion of derivatives of labelled amino acid. Contributions from mass labelled species to the total EC levels detected being generally consistent with those expected for natural abundance (1.11 % for ^{13}C and 0.36% for ^{15}N [CRC Handbook of Chemistry and Physics 1982]).

From the results of fermentations C and D (sampling 3) an increase is shown in both total potential EC and that proportion of the total that derives from ^{15}N labelled aspartic acid added towards the end of fermentation. The amounts of ^{15}N potential EC detected when such must is allowed to continue fermenting to dryness increase slightly when compared to anticipated natural abundance levels. This suggests that the addition of a fresh nitrogen source at the end of fermentation, for example in the correction of a "stuck" fermentation, might stimulate production of EC precursor which is derived not only directly from the added source but also via other components of the medium.

Two recognised microbial sources of EC precursor in wine are (i) yeast arginase activity - in which arginine is degraded to urea (which may react with ethanol to produce EC) and ornithine (Ough *et al* 1988) and (ii) the arginine deiminase pathway of lactic acid bacteria (Liu *et al* 1994, 1995) in which citrulline is the proposed EC precursor. The results of the present study show that supplementation with labelled assimilable nitrogen source (under conditions prevailing as fermentation approaches dryness) stimulates precursor formation. Whilst there appears to be a dramatic increase in total potential EC, ^{15}N labelled EC represents less than 1% of this total. From this we see that the effect of metabolism of residual nitrogen towards the end of fermentation upon total potential EC certainly requires further study. In conclusion it appears that the intracellular contents at the time of inoculation, i.e. that which is determined by pre-culture conditions, has no significant influence upon total EC precursor levels.

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