Diversity in Spoilage Yeast Dekkera/Brettanomyces bruxellensis Isolated from French Red Wine. Assessment During Fermentation of Synthetic Wine Medium

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JIB 526 Diversity in spoilage yeast *Dekkera/Brettanomyces bruxellensis* isolated from French red wine. Assessment during fermentation of Synthetic Wine Medium.

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ABSTRACT

Yeast *Brettanomyces bruxellensis* is a contaminant found worldwide and is responsible for red wine spoilage due to the development of animal and phenolic off-odours. During this study, 24 *Brettanomyces bruxellenis* isolates were obtained from red wine samples from two French wineries and these were discriminated as 23 strains. Nine strains coming from 2 wineries and 4 vintages were cultivated in synthetic wine medium during 1500 hours and they gave nine different behaviours. Four main growth patterns (with different growth steps and durations) and three main different sugar consumption profiles were obtained. Glucose and fructose were not a limiting substrate for all strains. The production level of 4-ethylphenol was found to vary from strain to strain (from 0.35 to 2.773 mg L^{-1}) and was independent of biomass concentration. Some strains presented a coupled-to-growth production of volatile phenols, others did not. This study showed that different strains of *Brettanomyces bruxellensis* behaved differently one from another under the given conditions taking into consideration several aspects. The results thus demonstrate a huge intraspecific diversity.

KEYWORDS: *Brettanomyces bruxellensis*, diversity, wine, growth profiles, 4-ethylphenol.

INTRODUCTION

The genus *Dekkera,* and its asporogenous form *Brettanomyces,* are well recognized as spoilage yeasts of industrial alcoholic fermentations[9,10] and fermented beverages such as beer and wine[5,14,22,23]. In red wine this contaminant is responsible for odour and taste defaults qualified as horsy, sweaty and mousy[2]. These unpleasant aromas are due to volatile phenols such as 4-ethylphenol and 4-ethylgaiacol[2] and other volatile compounds, including tetrahydropyridines (the "mouse taint"). *B. bruxellensis*, the main species naturally present in the wine environment[27,35], has been shown to synthesize the most important quantities of these phenolic volatile compounds, from the natural hydroxycinnamic acids present in grape musts: namely para-coumaric and ferulic acid[11,21]. These faults cause large economical losses[13]. In the last fifteen years, studies regarding these microorganisms have multiplied in a significant way[2, 7, 20, 31, 35] and indicates a desire to understand the behaviour of *Brettanomyces* and to prevent or at least control its development in the winemaking process.

Nevertheless, if one tries to synthesize and summarize the different works that have been produced regarding *Brettanomyces* in winemaking, it seems that accurate and definitive conclusions cannot be generalized given that each work has been performed with different strains from private collections. Two questions arise. Do all strains have similar behaviours and also whether in a given cellar? Does a dominant contaminant strain for a given vintage exist? Little data are available in literature regarding these points. Two studies have compared isolates from different wine regions, but not from the same cellars[7,8]. Our study thus consisted of screening *B. bruxellensis* strains isolated from wines coming from two cellars in order to determine if they had similar or

different characteristics, and focusing on their behaviour during fermentation on synthetic wine medium.

MATERIALS AND METHODS

Microorganisms.

Isolation: Strains of *Brettanomyces* used in this study (designated as strains B to Z) were isolated from red wines. The isolation procedure consisted of micro-morphological and physiological assessments, using selective media (testing resistance to cycloheximidine, acetic acid production, and conversion of p-coumaric into volatile phenols). Strain A was a reference strain isolated from a distillery plant[37] and identified by the Institute of Hygiene and Epidemiology of Brussels (THEM) as *B. bruxellensis* strain 6037. A PCR assay, using primers designed to the 26S rRNA gene was used to confirm that the isolated strains belonged to *B. bruxellensis* species[29]. Specific primers were:

DBRUXF: 5'-GGATGGGTGCACCTGGTTTACAC-3'

and DBRUXR: 5'-GAAGGGCCACATTCACGAACCCCG -3'.

Molecular typing by PCR Internal Transcribed Spacer (ITS), Enzymatic Restriction (ER) and Pulsed Field Electrophoresis (PFE): After DNA isolation and dilution[30], the rRNA coding region was amplified with primer pairs ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3')[19]. PCR products were digested with restriction enzymes *Hinf*I and *αTaq*I. Restriction segments were pulsed-field-electrophoresed and ethidium bromide was used for staining before photography.

Media and culture conditions.

Strain Conservation: All strains were stored at 4°C on Petri dishes containing a medium consisting of 10 g of glucose, 5 g of yeast extract (Oxoid,UK), 20 g of agar and 1 L of H_20.

Preculture: A two step preculture was performed for activation and acclimatation. The first one was carried out in 150 mL Erlenmeyer flasks with 75 mL of liquid medium [PreLM1] consisting of : 10 g of glucose, 10 g fructose, 1 g yeast extract, 2 g $(NH_4)_2SO_4$, 5 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 3 g tartaric acid, 3 g malic acid, 0.3 g citric acid and 1 L of H_20. After inoculation by a single colony from an agar plate, each flask was incubated at 30°C and magnetically stirred at 150 rpm for 72 h using a magnetic stirrer. The second preculture was inoculated at 10^6 cells mL^{-1}, incubated at 20°C and stirred at 150 rpm. For this step, 250 mL Erlenmeyer flasks with 150 mL of [PreLM2] medium were employed, similar to [PreLM1] but with 4 % (v/v) ethanol. This preculture was used for inoculating the main fermentation, when it reached the mid-exponential growth phase.

Fermentations: Fermentations were carried out in 500 mL Erlenmeyer flasks with a 300 mL initial working volume of Synthetic Wine Medium [SWM] containing the following: 2 g glucose, 8 g fructose, 0.5 g yeast extract, 0.4 g $(NH_4)_2SO_4$, 5 g of KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 3 g tartaric acid, 3 g malic acid, 0.3 g citric acid, 6 g glycerol, 5 mg p-coumaric acid, 10% (v/v) ethanol and 1 L of H_20.. Batch fermentations were inoculated with 10^5 cells mL^{-1} and were incubated at 20°C with shaking at 150 rpm.

The initial pH of all liquid cultures was adjusted to pH 3.4 with 10 M NaOH. All liquid media were sterilized at 120°C for 30 min (when containing ethanol, this was added

after autoclaving and the ethanol was previously sterilized by filtration through a 0.2 µm porosity filters).

The reproducibility of the fermentations was tested by conducting the B strain experiments in triplicate under standard conditions. The standard deviation was less than 10% for fermentation duration, biomass dry weight, sugars and ethanol concentration measurements.

Analytical techniques.

Microorganisms. Growth was followed by numeration (cells mL^{-1}) using a Thoma hemacytometer and an Olympus BH-2 microscope – x 400 enlargement. Standard deviation was less than 13 % for all cultivated strains. Final biomass concentration was also determined as dry weight, using a gravimetric method. To determine dry weight, 10 mL of culture sample was centrifuged for 10 min at 2500 rpm. The cells were washed twice with distilled water and dried using a humidity analyser (model HA60, Precisa, Zurich, Switzerland).

Sugars. Total sugar concentration was determined using the dinitrosalicylic colorimetric method[25]. Glucose was assayed with an enzymatic glucose analyser 2700 Select (YSI, Yellow Springs, Ohio) using glucose-oxydase.

4-ethylphenol. The concentration of 4-ethylphenol was measured by headspace Solid-Phase Micro-Extraction coupled with Gas Chromatography and Flame Ionization Detection[27]. Calibrations were performed in the synthetic wine medium. The standard deviation was less than 6%.

RESULTS

Genetic identification and typing.

Brettanomyces strains used in this study (designated as strains B to Z) were isolated from post alcoholic fermentation red wines, sampled from two cellars in the south-western region of France (Madiran and Buzet) between 2001 and 2004 (Table I). The first PCR step confirmed that all 24 isolates belonged to the *Brettanomyces bruxellensis* species, validating the isolation procedures (results not shown).

Further genetic typing using the PCR ITS and an enzymatic restriction (*Hinf*I and α*Taq*I) coupled with PFE was performed in order to screen the isolates at an intraspecies level. Electrophoresis profiles are presented in Fig. 1 and there were no strict identical isolates with the exception of two strains. Strains B and M appeared to have exactly the same profile. Since they were isolated from the same sample, we can assume that the 2 isolates are the same strain.

Nine strains were selected from the 23 identified. To compare their behaviour during fermentation, two strains (D and H) among the 10 strains isolated from Madiran Winery and seven strains (B, O, Q, S, V, W and X) among the fourteen from the Buzet winery, were chosen arbitrarily from both vintages.

Behaviour during fermentation.

In order to control the parameters, the experiment was performed with a synthetic wine medium [SWM]. To test the strains in an enology context, the synthetic medium was close to a wine at the final phase of alcoholic fermentation (but without any polyphenols). Total cell numeration was chosen to avoid under evaluating the population in regard to cells in a viable but non-cultivable state (viable and active cells that are not able to grow on classical solid medium), since this is often the case in long

term cultures and under stressful conditions such as in alcohol-containing medium[7,25], Moreover high metabolic activity was noted until the end of the experiments.

Growth profiles. Fig. 2 shows the growth profile comparison for the strains and indicates that there was no unique profile. Even if all of the strains had presented a long lag phase (the shortest being strain X with 200 h) and despite an inoculation from a preculture in mid-exponential growth phase, the profiles differed for both the kinetics and the population levels. The four main growth patterns could be differentiated.

Type 1: a single growth period with a long stationary phase after an exponential growth phase (strains B and W).

Type 2: two growth periods. A first phase with a classical exponential growth period followed by a constant and almost linear second growth phase (strains D and Q).

Type 3: three periods. A first phase with an exponential growth period, followed by a relatively long stationary phase, then a second growth with another exponential phase (strains H and S).

Type 4: close to Type 3, but with a second growth phase that was not exponential, it was slower (strains O, V and X).

Sugars consumption. As shown in Fig. 3, several behaviours were detected for the sugar consumption profiles.

Type 1: the rapid exhaustion of both glucose and fructose in one phase ; with strains B, O and X there was a very fast utilization of the sugars, strain Q was slower.

Type 2: a very slow and progressive consumption of sugars; strain D to 0 g L^{-1}, while strain W was unable to consume all the initial sugar and left 3.4 g L^{-1} of residual sugar.

Type 3: a two step consumption of sugars, separated by a long stationary phase. At the end of fermentation the concentration was 0 g L^{-1}; strain S entered stationary phase at 6 g L^{-1} at 500 h and strain V at 500 h was at stationary phase with 0.5 g L^{-1}. Strain H was classified in the last group with a slow first step sugar consumption, followed by a short 250 h stationary phase, and then a second sugar consumption to a residual concentration of 0.2 g L^{-1}.

All nine strains consumed both glucose and fructose. While glucose was always depleted before 1400 h, fructose was not depleted with strains H and W. Moreover, all strains started consuming glucose before fructose, but fructose consumption always started before glucose totally disappeared from the medium (results not shown). For strains B, O, Q and X, the total depletion of sugars occurred simultaneously to the first growth phase (Fig. 2 and Fig. 3).

4-ethylphenol profiles. Table II shows significant differences between the nine strains in term of 4-ethylphenol production. Both the maximal concentration (from 0.35 mg L^{-1} to 2.773 mg L^{-1}) and the time necessary to reach this level differed from one strain to the next (from 600 h to 1400 h with the majority at 1300 h). Moreover, when growth profile is superimposed on the 4-ethylphenol production pattern, several observations can be made. Indeed, some strains such as B, W and X appeared to have a direct "coupling" between growth and 4-ethylphenol production as shown in Figure 4.A. for strain B. But for the other strains (H, O, S, V, X) the production of 4-ethylphenol was detected without observing any growth between 500 and 900 h (as illustrated for strain V on Fig. 4.B.). Nevertheless the production of 4-ethylphenol during the stationary growth phase was very weak for strains O and X (data not shown). On the

opposite, strain Q presented significant growth without significant production of 4-ethylphenol between 300 h and 500 h of culture (Figure 4.C.).

DISCUSSION

Except for strain B also referenced as M, the genetic screening of the 24 isolates showed 23 different ER-PFE profiles, implying that 23 different strains of *Brettanomyces bruxellensis* were present. No prevalent strain was identified from either a given site or for a given vintage. From a given cellar and during one vintage, isolates coming from different tanks (five from Madiran 2001, five from Madiran 2002, six from Buzet 2003 and eight from Buzet 2004) were identified as different strains, proving the richness of the contamination pool. Research by de Souza et al.[33] has also shown intraspecific variability among industrial isolates contaminating one bio-ethanol plant. On the contrary, Mitrakul et al.[26] suggested the predominance of a single strain on spoiled wines from several vintages produced in a California winery. Our observations suggest that different kinds of contamination (single or multiple strain/s) may occur depending on the winery. Moreover one can hypothesize that the origin of the contamination could be different according to the tanks, and coming not only from the cellar, but for example, also from the vineyard. However, the genotype of the isolates was not further studied. In a recent publication[8] it was reported that 244 isolates from 31 winemaking regions in Australia, resulted in 8 genotypes being found, suggesting common points of origin of some strains.

In the second part of this study, nine strains were tested during the fermentation of synthetic wine medium, and nine different profiles were obtained, reflecting a significant diversity in metabolic and physiological behaviour. All strains presented a

very long lag phase, and some strains presented a stationary period between two growth steps. The existence of such long periods, without any noticeable population increase, before significant growth must be taken into account during monitoring in winemaking. It suggests that a low cell concentration of *Brettanomyces* observed on one day, which would suggest a no-risk situation in short term, might result in high contamination on the following days, leading to spoilage if preventive procedures and care are not taken.

Among the nine growth profiles four types were distinguished. One could hypothesize what might have occurred during the different growth steps. For growth with one single step, such as with strain B, a main substrate could be converted into biomass. Comparisons between the growth profile and sugar consumption profile clearly identified the sugars [glucose + fructose] as the limiting substrate. Once these substrates were depleted, the strain was unable to use the other substrates to increase its population. Strain W also had single growth period, but it behaved differently in its sugar consumption. Sugars were progressively depleted both during growth and the stationary phase. A limiting substrate, different from sugars, could cause the growth stop. For two step growth profiles, with a linear second phase (growth pattern of type 2) one can propose that it was a limitation of several necessary compounds (or at least one). These compounds may be provided progressively in limited proportions, for example dissolved oxygen. Growth profiles of type 3 appear like diauxic profiles. Indeed previous works have reported that yeasts of the genera *Dekkera/Brettanomyces* were able to shift from glucose, to a secondary substrate, to start a second growth phase. The two growth phases were separated by a new latent period, necessary for enzymatic adaptation. Such diauxic growth has been described when specific sugars, [glucose + sucrose] or [glucose + cellobiose], were used as the carbon source[1, 15, 18], with glucose

always being consumed first. Glucose and fructose were the only sugars used in the medium for the experiments reported here and the diauxic profile did not match with a sequential consumption of these two sugars. It appeared that for strains H, S and V, the two phases of sugars consumption (both glucose and fructose), were separated by a constant sugar concentration phase, and were in parallel with the two phases of growth (Fig.2 and 3). These three strains appeared to use the sugars for the two steps of growth. Until now, no clear explanation can be proposed for the two separated-phase phenomenom. One can assume that a compound necessary for growth was in default and that the yeast readapted its metabolism before pursuing growth (eg. nitrogen sources have to been known to have a limiting compound effect). Growth profiles of type 4, could be explained as a combination of diauxy and a limitation during the second growth phase. Sugars were depleted during the first growth step of strains O and X. and their stationary phase lasted over 500 h, before there was a new slow and progressive increase in cellular concentration.

For all strains examined, glucose was always consumed before fructose, but fructose was consumed while glucose was still available at low concentrations. Such observations were also made by Gilis[15] who suggested a competition between these two sugars for the same transporter. Other authors have demonstrated the co-existence of two transport systems for glucose[34].

The different hypotheses proposed suggest the existence of other substrates that have not yet been identified. Ethanol is well known for being a carbon[32] and energy source for *Brettanomyces spp.* under both aerobic and semi -aerobic conditions[12, 16]. Cotton-plugged and shake flask fermentations would be considered as micro-aerobic, resulting in favorable conditions for ethanol reduction and acetic acid production[6]. But in this case, ethanol analysis (data not shown) showed that ethanol was consumed by all the strains in the second part of fermentation (13 to 25 g/L) without any correlation to the second growth phase when it existed.

Moreover results from Table III lay the emphasis on significant diversity with 4-ethylphenol production and show important differences between the strains, both in terms of the maximal amount of volatile phenol and the time necessary to reach this concentration. The biotransformation leading to volatile phenol synthesis implies two enzymatic reactions: the first step is a decarboxylation of the phenolic acids into vinyl phenols, the latter being transformed during the second step into ethyl phenols by a vinyl reductase. The second reaction is very active in *B. bruxellensis*, compared to other yeasts or bacteria present in a wine related environment[11]. Investigating p-coumaric acid metabolism by this yeast, the results suggest that the conversion is not directly linked to the metabolism pathways for growth (e.g, strains produce 4-ethylphenol during the non-growth phase). It is also important to note that the highest 4-ethylphenol production was not linked to the highest cell concentrations (Table II). For instance, strain W had a maximum population of 0.5 $g.L^{-1}$ and produced 2.749 $mg.L^{-1}$ of 4-ethylphenol, while strain H population reached 2.6 $g.L^{-1}$ and produced 0.35 mg L^{-1} of 4-ethylphenol. *Brettanomyces* strains appear to have a different specific efficiency for converting p-coumaric acid. This result was consistent with a study by Conterno et al[8] who showed that one of the most discriminating characteristics for physiological diversity among 47 strains was the production of 4-ethylphenol. A study comparing 6 strains[14] pointed out that significant variation was noted for growth rate, final population level and 4-ethylphenol production. Similar observations have also been reported from wineries. Some spoiled wines present high concentration of 4-ethylphenol, but a relatively low cell concentration of *Brettanomyces* (suggests there are high producing strains), while others have a high *Brettanomyces* population but relatively low 4-ethylphenol quantities and no “Brett taint” (suggests low producing strains). Consequently, a low level of

contaminant in the wine may not prevent from the wine spoiling if the implicated strain is a strong 4-ethylphenol producer.

A major expectation for winemakers is to have effective tools in hand to prevent and control any risk of microbial spoilage, especially regarding *Brettanomyces*. This study shows that it would be hasty to draw conclusions from experiments with only one or two strains as being accurate and representative since strains appear to behave differently one from another, thus reflecting the huge diversity amongst the species. This diversity also recently been reported by Conterno et al.[8] for other characteristics, such as potential substrates, and it makes it more difficult to propose a curative method to protect against the risk of *Brettanomyces* contamination.

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LEGENDS

FIGURES

Fig. 1. *Hinf*I and *αTaq*I RE analysis of the PCR product obtained using specific pair primers ITS1 and ITS4. lad: molecular weight marker (100-bp ladder, Sigma), A: reference strain 6037, B to Z : isolated strains.

Fig. 2. Growth profiles for nine strains of *Brettanomyces bruxellensis* during fermentation of synthetic wine medium at 20°C.

Figure 2.A: Strains O (Buzet 2003), strains W and X and S (Buzet 2004).

Figure 2.B: Strains B, Q (Buzet 2003), H (Madiran 2002), V (Buzet 2004) and D (Madiran 2001).

Fig. 3. Total sugar utilization by nine strains of *Brettanomyces bruxellensis* during fermentation of synthetic wine medium at 20°C.

Figure 3.a: Sugar for strains O (Buzet 2003), strains W and X and S (Buzet 2004).

Figure 3.b: Sugar for strains B, Q (Buzet 2003), H (Madiran 2002), V (Buzet 2004) and D (Madiran 2001).

Fig. 4. Growth profiles and 4-ethylphenol production by three strains of *Brettanomyces bruxellensis* during fermentation of synthetic wine medium at 20°C

TABLES

Table I. *Brettanomyces* isolates used in the study: origin and campaign.

Table II. Maximal 4-ethylphenol concentration and associated time and biomass concentration.

FIGURES

1-

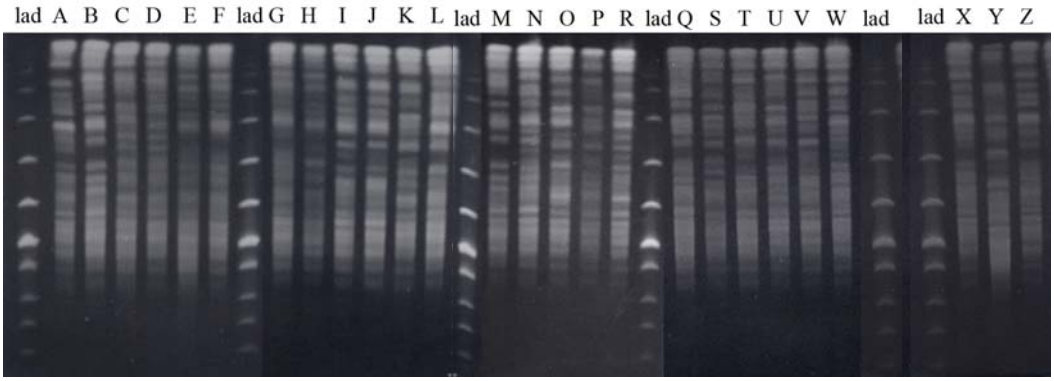

Figure 1. *Hinf*I and *αTaq*I RE analysis of the PCR product obtained using specific pair primers ITS1 and ITS4. lad : molecular weight marker (100-bp ladder, Sigma), A: reference strain 6037, B to Z : isolated strains.

2 -

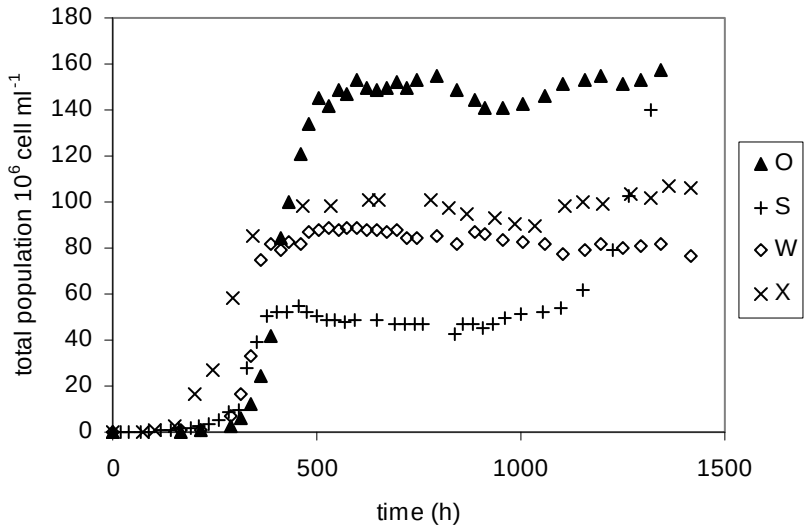

Figure 2.A: Strains O (Buzet 2003), strains W and X and S (Buzet 2004).

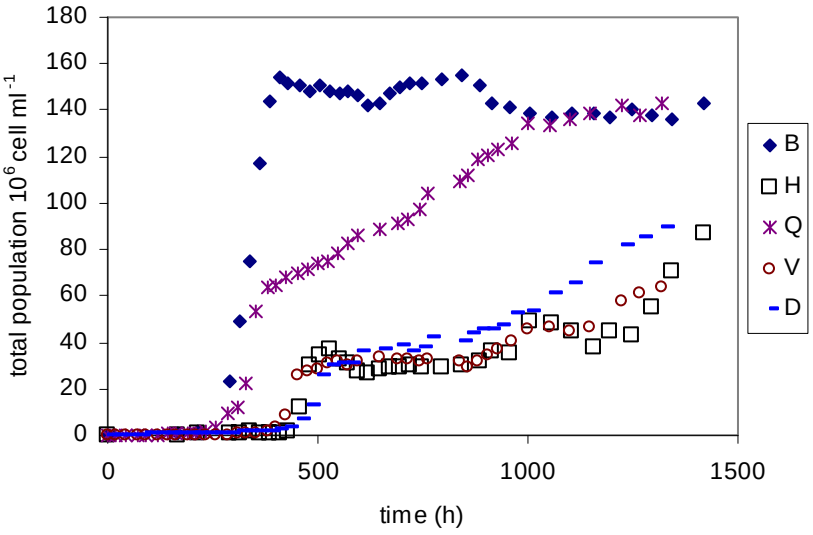

Figure 2.B: Strains B, Q (Buzet 2003), H (Madiran 2002), V (Buzet 2004) and D (Madiran 2001).

Figure 2. Growth profiles for nine strains of *Brettanomyces bruxellensis* during fermentation of synthetic wine medium at 20°C.

3 -

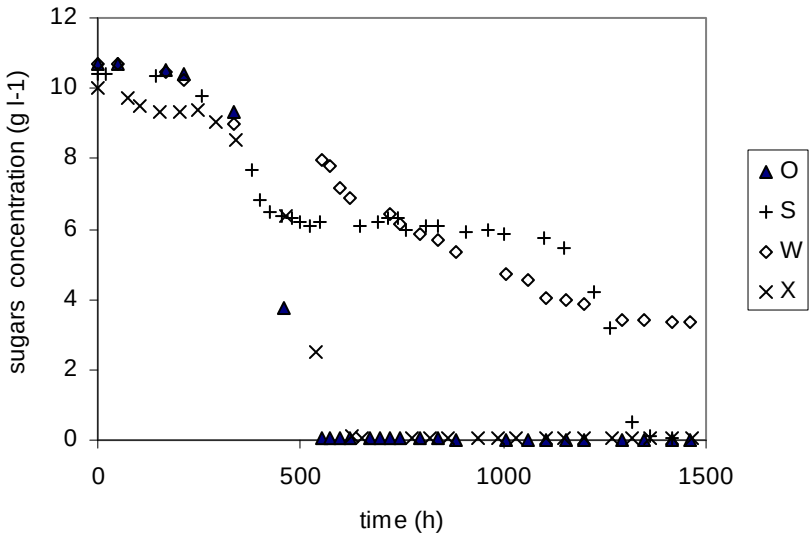

Figure 3.a: Sugar consumption by strains O (Buzet 2003), strains W and X and S (Buzet 2004).

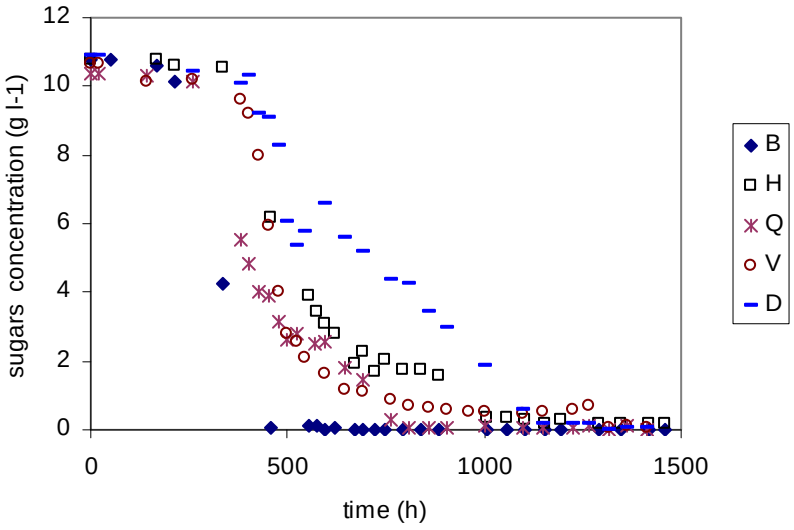

Figure 3.b: Sugar consumption by strains B, Q (Buzet 2003), H (Madiran 2002), V (Buzet 2004) and D (Madiran 2001).

Figure 3: Total sugars consumptions by nine strains of *Brettanomyces bruxellensis* during fermentation of synthetic wine medium at 20°C.

4-

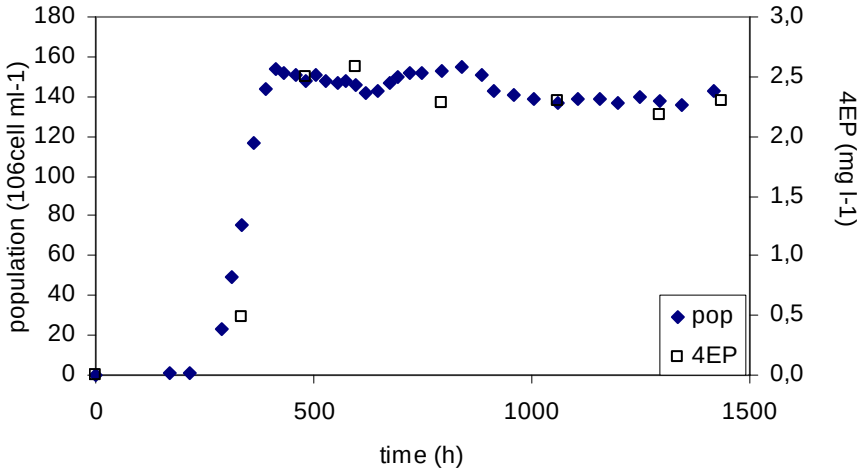

Figure 4.a: Strain B.

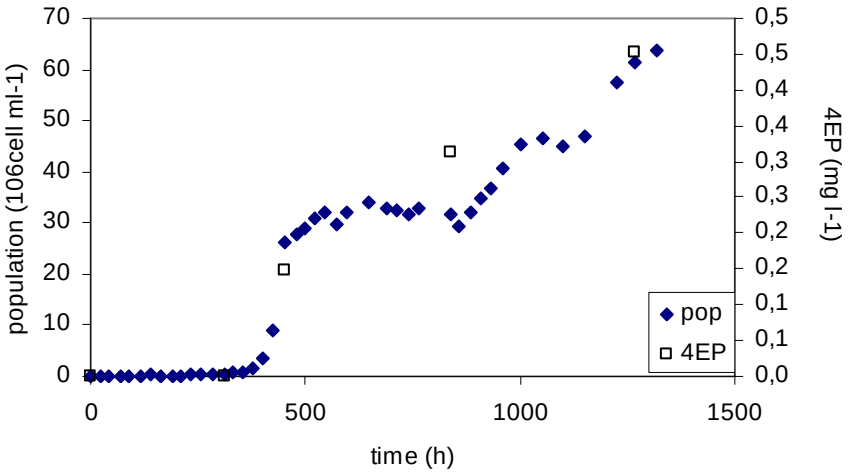

Figure 4.b: Strain V.

Superscripts for 6 and 1

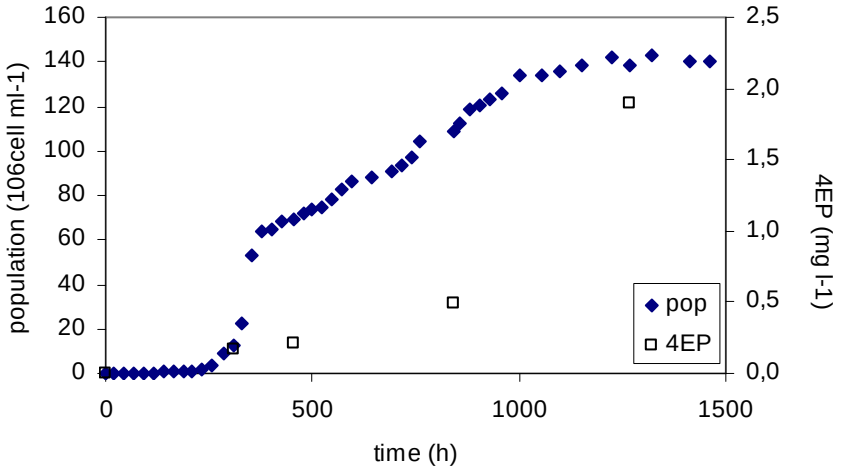

Figure 4.c. Strain Q.

Figure 4. Growth profiles and 4-ethylphenol productions by three strains of *Brettanomyces bruxellensis* during fermentation of synthetic wine medium at 20°C.

Table I. *Brettanomyces* isolates used in the study: origin and year

Strains	Origin	Year
A	Distillery	1997
B	Buzet	2003
C	Madiran	2001
D	Madiran	2001
E	Madiran	2001
F	Madiran	2001
G	Madiran	2001
H	Madiran	2002
I	Madiran	2002
J	Madiran	2002
K	Madiran	2002
L	Madiran	2002
M	Buzet	2003
N	Buzet	2003
O	Buzet	2003
P	Buzet	2003
Q	Buzet	2003
R	Buzet	2004
S	Buzet	2004
T	Buzet	2004
U	Buzet	2004
V	Buzet	2004
W	Buzet	2004
X	Buzet	2004
Z	Buzet	2004

Table II. Maximal 4-ethylphenol concentration and associated time and biomass concentration.

Strains	Cmax (mg/L)	Biomass (g/L)	Time (h)
B	2.59	1.88	600
D	2.773	1.35	1300
H	0.35	2.6	1400
O	2.762	1.55	1300
Q	1.895	1.54	1300
S	2.022	0.68	1300
V	0.453	2.08	1300
W	2.749	0.5	800
X	1.228	0.94	1400