RESEARCH ARTICLE

Cinnamic acid, ethanol and temperature interaction on coumarate decarboxylase activity and the relative expression of the putative *cd* gene in *D. bruxellensis*

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Abstract *Dekkera bruxellensis* is one of the main contaminating yeasts in wine due to its ability to metabolize cinnamic acids into volatile phenols. This yeast metabolizes *p*-coumaric acid into 4-vinylphenol through a coumarate decarboxylase (CD) and then transforms it into to 4-ethylphenol (EF) through a vinylphenol reductase. In this work we investigated the influence of the interaction between the concentration of *p*-coumaric acid, ferulic acid and ethanol as well as growth temperature on the production of CD activity and the expression of a putative gene that codes for this enzymatic activity. For this, a Box Behnken experimental design was used. The concentration of *p*-coumaric acid (5-26 ppm) and ferulic acid (3-9 ppm) alone did not show any significant effect on any of the studied response variables. However, the interaction between (ethanol concentration * cinnamic acid concentration) and (ethanol concentration * temperature) had a significant statistical effect on the production of CD activity. Additionally, a higher growth temperature negatively affected the expression of the putative *cd* gene and the production of CD activity. This is the first work that studies the effect of cinnamic acids on the production of CD activity and the relative expression of its putative gene, using natural concentrations of cinnamic acid found in wine.

INTRODUCTION

The genus *Dekkera* has been described in almost all wine-producing areas (Chatonnet et al. 1992; Rodrigues et al. 2001; Loureiro and Malfeito-Ferreira, 2003; Ganga and Martínez, 2004; Conterno et al. 2006; Suárez et al. 2007). The presence of this yeast in wine is associated with phenolic aromas that negatively influence the sensorial characteristics of the product. The formation of these off-flavours is due the metabolization of cinnamic acids, present in the must, which give rise mainly to 4-ethylphenol (EF) and 4-ethylguaiacol (Chatonnet et al. 1992). The cinnamic acids, in special *p*-coumaric and ferulic acids, are first decarboxylated to vinyl derivatives by a cinnamic decarboxylase (CD) and then reduced to ethyl derivatives through the action of a vinylphenol reductase (VR) (Chatonnet et al. 1992). Cinnamic acids have an anti-microbial function and therefore all microorganisms that ferment plant products have this type of enzymatic activity (Clausen et al. 1994; Cavin et al. 1997; Cavin et al 1998; Coghe et al. 2004). The majority of yeast species isolated from wines are capable of producing 4-vinylphenol from *p*-coumaric acid (Chatonnet et al. 1992; Dias et al. 2003), but few can metabolize *p*-coumaric acid into 4-EF (Chatonnet et al. 1992; Barata et al. 2006; Lopes et al. 2009). Works on *D.*

bruxellensis have mainly focused on its early detection to reduce economic losses (Wedral et al. 2010). Likewise, studies have been carried out to understand the mechanisms of 4-vinylphenol and 4-EF production (Dias et al. 2003; Godoy et al. 2008; Harris et al. 2008; Harris et al. 2009). It has been observed that when *D. bruxellensis* grows in the presence of cinnamic acids, especially *p*-coumaric acid, CD activity increases 600 times (Godoy et al. 2008). Higher EF production occurs when the yeast is grown with a lower concentration of ethanol, given that with 15% of ethanol EF production decreases drastically (Dias et al. 2003; Suárez et al. 2007). Likewise, growth temperature also affects the production of volatile phenols where a temperature of between 16 and 22ºC is favourable (Dias et al. 2003; Benito et al. 2009). However, all these factors have been studied separately without considering their interaction or the effect of cinnamic acids in concentrations naturally present in the must.

A partial identification of the gene that codes for CD activity in *D. bruxellensis* has been described (Harris et al. 2009). In the case of bacteria, the *pdc* gen of *Lactobacillus plantarum* which codes for a *p*-coumarate decarboxylase has also been described. This gene is regulated by the presence of a substrate, the activity of which is 6000 times greater in the presence of *p*-coumaric acid (Cavin et al. 1997). A similar observation was made with the *pad* gen that codes for a phenolic acid decarboxylase in *Bacillus subtilis* (Cavin et al. 1998) which is regulated at the transcriptional level. In *S. cerevisiae* regulation is at the post-trancriptional level (Clausen et al. 1994). The first enzyme that metabolizes the cinnamic acids in *D. anomala* was partially purified and has recently been partially sequenced (Harris et al. 2009). Our research group has purified and characterized a CD and vinyl phenol reductase from *D. bruxellensis* (Godoy et al. 2008). Using bioinformatic analysis our group obtained a partial sequence of the gene that would code for this enzymatic activity (putative *cd* gene). The objective of the present work was to determine the combined effects of the concentrations of cinnamic acids and ethanol at levels naturally found in wine, and growth temperature on CD activity in *D. bruxellensis* and the relative expression of the putative *cd* gene.

MATERIALS AND METHODS

Microorganism

D. bruxellensis L-2480 was isolated from a Chilean winery. This strain is maintained in the collection of the Biotechnology and Applied Microbiology Laboratory of the Universidad de Santiago de Chile (LAMAP-USACH).

Culture medium and growth conditions

The yeast *D. bruxellensis* L-2480 was grown in YPD medium (5 g L^{-1} yeast extract, 5 g L^{-1} peptone, 20 g L^{-1} glucose and 20 g L^{-1} agar) for four days at 28ºC. For the assays described in Table 1, flasks containing 300 ml of yeast nitrogen base (Difco, USA) 6.7 g L^{-1} and glucose 20 g L^{-1} were inoculated with 1 x 10^6 cells mL^{-1}. Cinnamic acid and ethanol concentrations were added according to Table 1. Each culture was agitated until a final concentration of 1 x 10^8 cells mL^{-1} was obtained.

Expression of the putative *cd* gen

Extraction of total RNA and cDNA synthesis. Total RNA extraction was carried out when the cultures reached a density of 1 x 10^8 cells mL^{-1}, using the method based on the RNeasy Mini Handbook (Qiagen, USA). Total RNA was subsequently used as a template for the reverse transcription reaction (RT) using the method described by Zhu and Altmann (2005).

Real time PCR (QPCR). The cDNA obtained was used as a template for QPCR. The primers used for the putative *cd* gene were CD-F (TCTTCCAAGCAGGGATTTTG) and CD-R (CATTCCGCCTCCACTTTTATC) and the *act* gene was used as a housekeeping gene. The QPCR were performed on a LightCycler 1.5 (Roche, Germany) with 10 µl of 2X Brilliant II SYBR Green QPCR Master mix (Stratagene, USA), 0.1 mg mL^{-1} of BSA (New England BioLabs, USA) and 0.5 µM of each primer in a final volume of 20 µl. The program used was 95ºC for 10 min, 30 cycles at 95ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec, a denaturing analysis at 95ºC for 0 sec, 65ºC for 15 sec and 95ºC for 0 sec with a temperature increase of 0.1ºC/sec, and a cooling stage at 40ºC for 30 sec. The results

were analyzed with the LightCycler 4.0 software (Roche, Germany) and quantification of the relative expression of the putative *cd* gene was carried out using the mathematical method of Pfaffl (2001).

Determination of protein and enzymatic assays

The protein concentration was determined using the method described by Bradford (1976), with bovine serum albumin as a standard. Determination of coumarate decarboxylase activity (CD) was performed as described previously by Godoy et al. (2008).

Table 1. Runs of Box-Behnken design.

Experimental Runs	Temperature (ºC)	Ethanol (%)	Coumaric acid (ppm)	Ferulic acid (ppm)	Log relative expresión *cd* gen	CD activity (U) *
1	28	3	15.5	6	0.68	7.3
2	22	6.5	5	3	0.37	8
3	22	6.5	26	3	0.66	7.3
4	16	6.5	5	6	0.99	21.6
5	28	6.5	15.5	9	-0.72	9.3
6	22	6.5	26	9	0.3	9.4
7	16	10	15.5	6	1.00	33
8	22	3	5	6	1.47	8.1
9	22	10	26	6	0.32	13.7
10	22	10	15.5	3	0.07	9.7
11	16	6.5	26	6	1.49	27
12	22	3	15.5	3	0.86	8.8
13	22	6.5	15.5	6	0.46	12.7
14	22	6.5	15.5	6	0.65	11
15	22	6.5	15.5	6	0.56	13.7
16	28	6.5	15.5	3	-0.55	11.7
17	28	6.5	26	6	-0.55	5.4
18	22	3	15.5	9	0.80	7.8
19	28	10	15.5	6	-0.70	2.9
20	28	6.5	5	6	-1.70	14.5
21	22	3	26	6	1.35	8.8
22	22	10	5	6	-0.19	8.3
23	16	3	15.5	6	1.32	12.4
24	16	6.5	15.5	3	1.19	25.1
25	16	6.5	15.5	9	1.63	36.7
26	22	6.5	5	9	0.60	9.4
27	22	10	15.5	9	0.02	8

Effect of enological factors on the enzymatic activity CD and relative expression of the putative *cd* gene. Response surface design

A Box Behnken surface design was used to study the effects of concentrations of *p*-coumaric and ferulic acids, ethanol and growth temperature on CD activity and the relative expression of the putative *cd* gene. The levels of each variable under study are described in Table 1. The concentrations of *p*-coumaric and ferulic acids used for our assays were determined by quantifying these acids in Chilean wines of the Cabernet Sauvignon variety (data not shown). This design allowed for assaying the four factors and their interactions in a 24-set block with the experimental conditions and three central points. The experiments were conducted at random according to an order provided by the Statgraphics 16 program. The same program was used to analyze the experimental design (StatPoint Technologies Inc, 2009).

RESULTS

To determine how the interaction of temperature and ethanol, *p*-coumaric acid and ferulic acid concentrations affect the production of CD activity and the relative expression of the putative *cd* gene, a Box Behnken statistical experimental design was performed (Montgomery, 2001). The experimental runs and the results are shown in Table 1. This statistical tool allows for determining the effects of the factors under study with a minimum of experiments on the response variables, whether individually or considering them in interaction.

CD activity

The mathematical model of the quantifying CD activity provided by the experimental design explained 91.5% of the total variance (R^2 adjusted = 86.2%). The Analysis of Variance indicated those variables with significative effect on the production of enzymatic activity at its higher experimental levels Table 2. The simple effects plot Figure 1 shows that an increased ethanol concentration results in increased production of CD activity. However, above a concentration of 6.5%, CD activity production decays. Although the concentration of cinnamic acids alone does not influence the production of CD activity, the interaction between cinnamic acids and growth temperature has a negative effect on the production of this enzymatic activity (data not shown). Within the assayed conditions, the response surface methodology optimization routine found that the optimum conditions to maximize CD activity were temperature of 16ºC, 10% ethanol, 26 ppm of *p*-coumaric and 9 ppm of ferulic acid, resulting in 39U of enzymatic activity Figure 2a.

Table 2. Analysis of variance for CD activity.

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
A:Ethanol	41.813333	1	41.813333	4.33	0.0538
B:Temperature	913.5075	1	913.5075	94.66	0.0000
C:Coumaric acid	0.24083333	1	0.24083333	0.02	0.8765
D:Ferulic acid	8.3333333	1	8.3333333	0.86	0.3666
AA	57.041667	1	57.041667	5.91	0.0272
AB	156.25	1	156.25	16.19	0.0010
BB	267.6676	1	267.6676	27.74	0.0001
BC	52.5625	1	52.5625	5.45	0.0330
BD	49.0	1	49.0	5.08	0.0386
CC	13.425104	1	13.425104	1.39	0.2555
Total error	154.41333	16	9.6508333		
Total (corr.)	1821.0867	26			

R-squared = 91.5%.
R-squared (adjusted for d.f.) = 86.2%.

Relative expression of the putative cd gene

In this case, the Box Behnken design provided a goodness of fit of 87.1% (R^2 adjusted = 81.5% of explained variance) Table 3. Considering the F-ratio and *p*-values, increasing temperature and ethanol concentration had a negative linear effect on the relative expression of the putative *cd* gene Figure 3. Although the ferulic acid concentration has no statistically significant effect on the response, the simple effects plot shows that the concentration of *p*-coumaric acid had a slight positive effect on the expression of this gene. The response surface methodology optimization routine found the optimum conditions at 16ºC, 3% ethanol, 26 ppm of *p*-coumaric acid and 5 ppm of ferulic acid, which maximized the putative *cd* gene expression Figure 2b.
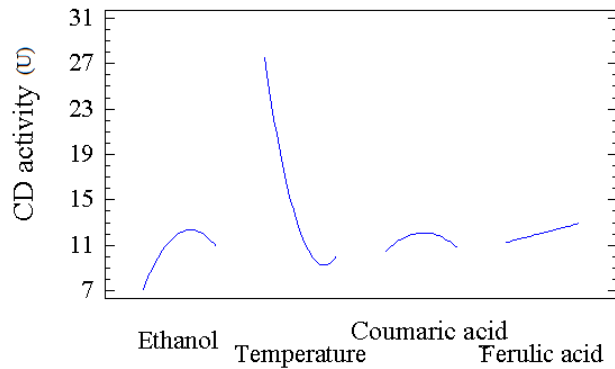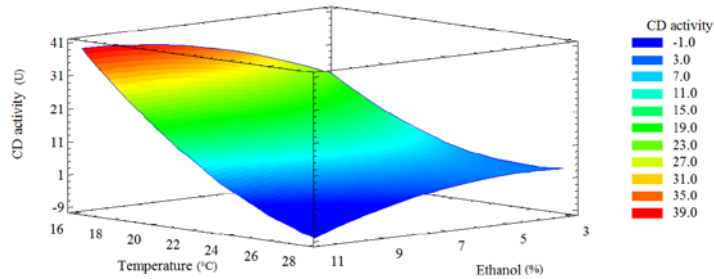

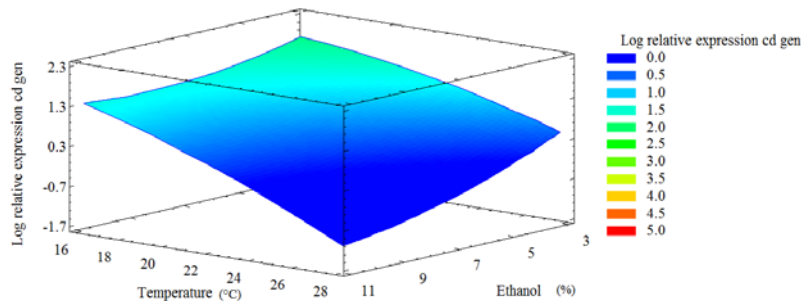**Fig. 1 Simple effects plot for CD activity.** (Y axis CD activity; X-axis variables under study).

a)

b)

Fig. 2 Response surface plots: a) CD activity; b) Log relative expression of putative *cd* gene.

DISCUSSION

Yeast of the genus *Dekkera/Brettanomyces* occurs mainly during aging in barrels (Benito et al. 2009). At this stage, the microorganism is under unfavourable conditions for its development, such as a low nutrient concentration, a high concentration of SO_2, acid pH and a temperature close to 15ºC (Suárez et al. 2007; Brandam et al. 2008, Renouf et al. 2009). Dias et al. (2003) described that a 5% ethanol concentration is adequate to obtain volatile phenols in the culture medium, since an increase in ethanol concentration is detrimental to the yeast population and consequently leads to a decrease in volatile phenols. In our study, all the cultures were grown to a final concentration of 1 x 10^8 cells mL^{-1}. However, our result is similar to that obtained by Dias et al. (2003), where although *D. bruxellensis* presents basal CD activity, it is necessary to add ethanol to the culture medium to increase its production Figure 1. In the case of the putative *cd* gene expression, an increased ethanol concentration has a negative effect on obtaining transcripts or their mean life. Works with *D. bruxellensis* have used concentrations above 100 ppm of *p*-coumaric acid in the growth medium, leading to higher production of volatile phenols, which in turn has been correlated with higher CD activity (Dias et al. 2003; Godoy et al. 2008; Harris et al. 2009). However, since this concentration of cinnamic acids has not been described in wines, we used concentrations known to be present in wine (Karathanos et al. 2008). We determined that under these conditions cinnamic acids do not significantly influence the production of CD activity. However, the interaction of cinnamic acids with growth temperature, and growth temperature with ethanol concentration, as well as ethanol concentration, are highly important variables in the production of CD activity. In the case of the expression of the putative *cd* gene, it was found that temperature and ethanol were the only variables that have a statistically significant effect on the response variable. Analyzing the assayed growth temperatures (16ºC to 28ºC) shows that the increase of this variable brings about a decrease of CD activity. This differs to that reported by Benito et al. (2009), who indicated that at a temperature of between 20 and 30ºC the yeast consumes the greatest quantity of *p*-coumaric acid, which is indirectly associated to the presence of higher CD activity production. Some authors have suggested that the presence of *p*-coumaric and ferulic acids in the culture medium of *B. bruxellensis* leads to an increase in descarboxylate hydroxycinnamic activity (Dias et al. 2003; Godoy et al. 2008; Harris et al. 2009). These observations suggest that the gene that codes for this enzymatic activity may be induced. In the case of *L. plantarum*, the *pdc* gene is inducible with *p*-coumaric acid, similar to what was observed for the *pad* gene of *B. subtilus* which is also induced by ferulic and caffeic acids. The concentration of cinnamic acids used by these authors was over 300 ppm, higher than the concentrations assayed in our work Table 1. All the works described to date have independently studied growth temperature, pH, and ethanol concentration, amongst others without considering their interaction. Through the experimental design used in this study, it was possible to determine the effect of each variable independently as well as their interaction and to define the optimum conditions for the production of CD activity and the expression of the putative *cd* gene. In general the values of the variable are similar, with the exception of ethanol concentration. Cavin et al. (1998) showed that *p*-coumaric and ferulic acids induce the expression of the *pad* gene of *B. subtilis*, obtaining maximum PAD activity at 10 min after induction. The *pad* gene will be transcribed as a monocistronic transcriptional unit and subjected to transcriptional regulation involving substrate-mediated induction. By studying the *pdc* gene of *L. plantarum* which codes for a *p*-coumaric acid decarboxylase. Cavin et al. (1997) showed that the *pdc* mRNA pool was at its maximum after 10 min of incubation with the substrate and decreased rapidly after *p*-coumaric acid was entirely metabolized. In all our experiments the *p*-coumaric acid was not metabolized completely with part of it remaining in the culture medium (data not shown). At 22ºC with 10% alcohol, the yeast on average only metabolizes 38% of the *p*-coumaric acid in the culture medium. While with 3% ethanol, 74% of the initial *p*-coumaric acid was metabolized by the yeast. This result was also obtained at 16ºC. Salameh et al. (2008) indicated that *p*-coumaric acid can react with the ethanol in the medium or be absorbed through the yeast wall, which leads to a decrease in the acid concentration in the culture medium. In our case, the lowest quantity of *p*-coumaric acid was obtained with 3% alcohol in the medium. The slow metabolization of *p*-coumaric acid in the culture medium is closely related to the growth rate of the yeast, with yeast growth slower at 10% than at 3%.

On the other hand, the culture medium used in this work had a pH of 3.4, at which level the cinnamic acids chemically not disassociated, as well as being lipophilic (Agnolucci et al. 2010). This allows these organic acids to enter the cell by diffusion and be deprotonated within the cell resulting in an acidification of the internal medium. To avoid this, the cell must metabolize them and eliminate the protons formed using a membrane ATPase (Agnolucci et al. 2010). Based on our results, alcohol plays an important role in the entrance of cinnamic acids. Sousa et al. (1996) described that in the case of yeasts, ethanol influences the uptake of the substrate from the culture medium. When the mechanism of removing the substrate is passive diffusion, increased ethanol concentration results in increased

consumption of the substrate while when the mechanism involves some type of transporter, ethanol has a negative effect. This suggests that in *D. bruxellensis* the transport of *p*-coumaric acid to the cell interior might be mediated by a transporter. Likewise, the presence of ethanol in the culture medium causes variation in the composition of the cellular membrane, which may influence the diffusion of cinnamic acids to the interior of the cell or on the ATPase itself or its surroundings (Chambel et al. 1999). However, more studies are necessary.

Table 3. Analysis of variance for Log relative expression of putative *cd* gene.

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
A:Ethanol	2.9313968	1	2.9313968	24.74	0.0001
B:Temperature	10.339777	1	10.339777	87.27	0.0000
C:Cumaric acid	0.350892	1	0.350892	2.96	0.1015
D:Ferulic acid	0.000147	1	0.000147	0.00	0.9723
AA	0.22201	1	0.22201	1.87	0.1870
AB	0.26988025	1	0.26988025	2.28	0.1477
BB	0.20164	1	0.20164	1.70	0.2076
Total error	2.2511595	19	0.11848208		
Total (corr.)	16.672713	26			

R-squared = 87.1%.
R-squared (adjusted for d.f.) = 81.5%.

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