

Interaction between *S. cerevisiae* MONA, PE-2, CAT-1 and ATCC in fermentation sugar cane broth

Interação entre *S. cerevisiae* MONA, PE-2, CAT-1 e ATCC em fermentação de caldo de cana-de-açúcar

Interacción entre *S. cerevisiae* MONA, PE-2, CAT-1 y ATCC en caldo de fermentación de caña de azúcar

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Abstract

The diversity microbial in ethanolic fermentation generate different behavior metabolic that depended on the microorganisms present. Some kinetic parameters can tell how interactions between microorganisms are occurring in fermentation and can also predict your metabolic behaviors. However, there are little studys about the influence of interactions microbial on kinetic parameters in fermentation sugar cane. Therefore, this work aimed to understand the influence of the yeast strain *Saccharomyces cerevisiae* CAT-1, MONA, PE-2 and ATCC in the production of biomass, ethanol, glycerol and sugar consumption, as well as to evaluate the kinetic parameters by means of response surface methodology for mixing. From the biomass models generated, it was observed that the yeasts ATCC and MONA when in the presence of CAT-1 and PE-2 showed antagonisms. For the ethanol, the synergistic effect was verified for the mixture MONA/ATCC and CAT-1/PE-2 being that CAT-1 and PE-2 were the yeasts that strongly favored the ethanol production. It stands out yeast MONA due to having lower glycerol production, character desirable in the sugar and alcohol industry. Thus, it is clear that from the analysis employed it was possible to infer about the kinetic behavior of the yeasts in pure cultures as well as the effect of the interaction between them during the cultivation.

Keywords: Ethanol; Co-culture; Metabolic behavior.

Resumo

A diversidade microbiana na fermentação alcoólica gera diferentes comportamentos metabólicos que dependem dos microrganismos presentes. Alguns parâmetros cinéticos podem dizer como as interações entre microrganismos estão ocorrendo na fermentação e também podem prever seus comportamentos metabólicos. No entanto, existem poucos estudos sobre a influência das interações microbianas nos parâmetros cinéticos na fermentação da cana-de-açúcar. Portanto, este trabalho teve como objetivo compreender a influência das estirpe de levedura *Saccharomyces cerevisiae* CAT-1, MONA, PE-2 e ATCC na produção de biomassa, etanol, glicerol e consumo de açúcar, bem como, avaliar os parâmetros cinéticos por meio da metodologia de superfície de resposta para misturar. A partir dos modelos de biomassa gerados observou-se que as leveduras ATCC e MONA quando na presença de CAT-1 e PE-2 apresentou antagonismos. Para o etanol, foi verificado o efeito sinérgico para a mistura das leveduras MONA / ATCC e CAT-1 / PE-2 sendo que CAT-1 e PE-2 foram as leveduras que mais favoreceram a produção de etanol. Destaca-se a levedura MONA por apresentar menor produção de glicerol, caráter desejável na indústria sucroalcooleira. Assim, percebe-se

que a partir da análise empregada foi possível inferir sobre o comportamento cinético das leveduras em culturas puras, bem como o efeito da interação entre elas durante o cultivo.

Palavras-chave: Etanol; Co-cultura; Comportamento metabólico.

Resumen

La diversidad microbiana en la fermentación de etanol genera diferentes comportamientos metabólicos que dependen de los microorganismos presentes. Algunos parámetros cinéticos pueden indicar cómo se producen las interacciones entre microorganismos durante la fermentación y también pueden predecir sus comportamientos metabólicos. Sin embargo, existen pocos estudios sobre la influencia de las interacciones microbianas sobre los parámetros cinéticos en la fermentación de la caña de azúcar. Por tanto, este trabajo tuvo como objetivo comprender la influencia de las cepas de levadura *Saccharomyces cerevisiae* CAT-1, MONA, PE-2 y ATCC en la producción de biomasa, etanol, glicerol y consumo de azúcar, así como evaluar los parámetros cinéticos mediante metodología de superficie de respuesta para la mezcla de estas levaduras. A partir de los modelos de biomasa generados, se observó que las levaduras ATCC y MONA cuando en presencia de CAT-1 y PE-2 mostraron antagonismos. Para el etanol, se verificó el efecto sinérgico en la mezcla MONA / ATCC y CAT-1 / PE-2 siendo que CAT-1 y PE-2 fueron las levaduras que favorecieron fuertemente la producción de etanol. Se destaca la levadura MONA por tener menor producción de glicerol, carácter deseable en la industria azucarera y alcohólica. Así, es claro que a partir del análisis empleado se pudo inferir sobre el comportamiento cinético de las levaduras en cultivos puros así como el efecto de la interacción entre ellas durante el cultivo.

Palabras clave: Etanol; Co-cultura; Comportamiento metabólico.

1. Introduction

Yeasts of genera *Saccharomyces. cerevisiae* are able to convert sugars into ethanol and carbon dioxide, as well as, in glycerol, acetate, succinate, pyruvate, higher alcohols and esters like response to environment through fermentative process (Jouhten et al., 2016; Parapouli et al., 2020). This biochemical process is responsible for supply of one of the more important biofuel, bioethanol, an attractive renewable fuel like alternative to the use petrol (Amorim et al., 2011). In relations to its production, various the researches have focused to increased efficiency through improvement sugar cane varieties, agricultural management, process engineering and fermentation, the becoming the theoretical sugar conversion into ethanol around of 90–92% (Lopes et al., 2016) .

In fermentations, besides to recycle 90 % of cells and to keep high cell density inside the fermenter, in industry are added intentionally yeast with high proven yield (Ceccato-Antonini & Covre, 2021; Lopes et al., 2016). In addition, native yeasts of cane sugar and contaminant bacteria are reported frequently. Consequently, the microflora associated with fermentative ecosystem is highly complex and composed for many interactions between yeast-yeast and yeast-bacteria (Brexó & Sant’Ana, 2018). All this diversity microbial in fermentation reflect in different behaviour the of microorganisms. It is known that the main pattern that detect the interactions between microorganisms during fermentations are those linked competition for nutrients, pH reduction, and syntheses of metabolites with ethanol and organic acids (Tosin & Andrietta, 2015). A few kinetic parameters also say how the interactions are occurring in fermentation. For example, behaviours synergic and antagonist can will detected by increment of biomass when compared pure culture and combined culture (Amorim et al., 2011). Through the kinetic parameters like fermentative efficiency and $Y_{P/S}$ too is possible say about the destine of carbon source.

Despite the existence of reports on the consequences of microbial interactions during bioethanol production, there is still little knowledge of the molecular mechanisms involved in the process between yeast-yeast, as well as, the relation the of effects of yeast-yeast interactions above fermentation kinetic parameters (Albergaria & Arneborg, 2016; Brexó & Sant’Ana, 2018). Therefore, understanding the modulations metabolic in the eco-physiological process fermentation may constitute a very useful tool to ensure the process is better controlled. A few examples of the advantages of this understanding could be the increase in ethanol yields due to the control of populations interactions.

Some research establishes a relation between the presence of contaminant bacteria and savage yeasts. In research of Souza et al. (Souza et al., 2012) was describes the effects of the presence of the yeast *Dekkera bruxellensis* and the bacterium *Lactobacillus vini* on the industrial production of ethanol from sugarcane fermentation. Theses contaminants were correlated to a decrease in ethanol concentration and the accumulation of sugar. It also was verified that the homofermentative *Lactobacillus plantarum* was more detrimental to industrial yeast strain (CAT-1) when compared with heterofermentative *Lactobacillus fermentum* due to reduced yeast viability and higher amount of lactic acid in the growth medium (Basso et al., 2014). Brexó and Sant'Ana (2018) reported strategies for survival by certain microorganisms, one of them is associated with increasing the concentration of ethanol, organic acids, and other extracellular metabolites. These strategies already are scientifically well established, however, there is still a gap about the physiological and molecular mechanisms governing these interactions.

In general content of articles is about the main contaminants of bioethanol (lactic acid bacteria) (Albergaria & Arneborg, 2016; Bassi et al., 2018; Beckner et al., 2011; Brexó & Sant'Ana, 2017, 2018; Lucena et al., 2010; Rich et al., 2015) , of which few report the interaction between yeast-yeast and its about ethanol production and other metabolite product. Other studies describe which yeast is present in alcoholic fermentation but neither describe the interaction between yeast-yeast from the kinetic point of view (Basso et al., 2008; Brown et al., 2013; Santos et al., 2017; Tosin & Andrietta, 2015).

This paper was understand the influence of each yeast *S. cerevisiae* CAT-1, MONA, PE-2 and ATCC on the production of metabolites (biomass, ethanol, glycerol) and consumed substrate, as well as, to determine kinetic parameters of culture with more of an yeast. It was used the design mixing with yeast concentration *S. cerevisiae* CAT-1, MONA, PE-2 and ATCC like independent variables and biomass, ethanol, glycerol, acetic acid and residual sucrose like dependent variables. It was also evaluated the fermentative parameters regarding biomass production (biomass conversion factor) and ethanol production (ethanol conversion factor, produdity and fermentative efficiency). These data were compared between pure cultures and cultures in mixtures for establishing a relation between metabolite formation and possible interactions among the yeasts studied.

2. Material and Methods

2.1 Strain of yeast and maintenance

For understanding the interaction between microorganism was used yeast *Saccharomyces cerevisiae* PE-2 (PE-2), *Saccharomyces cerevisiae* CAT-1 (CAT-1), *Saccharomyces cerevisiae* ATCC 7754 (ATCC) e *Saccharomyces cerevisiae* monastrell (MONA). The first two yeasts were kindly supplied by the Anicuns S/A Álcool e Derivados company, the third one was provided by the Coleção de Culturas Tropical da Fundação André Tosello (Campinas-SP, Brazil), and the last one was isolated from the wineries in Spain provided by the Instituto de Agroquímica y Tecnología de Alimentos (IATA). The yeasts were maintained in: (g.L⁻¹) YPD: (10) yeast extracts, (10) peptone, (20) glucose and (20) agar; YMA: (3) yeast extracts, (3) malt extract, (5) peptone, (10) glucose and (20) agar; and GPY: (5) yeast extracts, (5) peptone, (20) glucose and (20) agar, respectively. These same mediums were used to prepare the inoculum without agar. During the inoculum preparation, cultures were scrapped and placed in Erlenmeyer flask of 250 mL with 50 mL appropriate medium for 24 hours, at 28 °C, initial pH of 5.2 and stirring at 150 rpm.

2.2 Media and fermentation conditions

The fermentations were realized in a 250 mL Erlenmeyer flask and useful volume of 50 mL. The culture medium using the synthetic sugarcane juice was composed of (g.L⁻¹): glucose (10), fructose (10), sucrose (210), malic acid (1.8), citric acid (8), KH₂PO₄ (0.75), K₂SO₄ (0.5), MgSO₄.7H₂O (0.25), CaCl₂.2H₂O (0.16) and NaCl (0.2). In this solution was also added

36,5 mL of amino acid solution (composed by $\mu\text{g.L}^{-1}$): d-biotin (37.5), calcium-d-pantothenate (750), nicotinic acid (750), myo-Inositol (750), thiamine hydrochloride (750), piridoxal hydrochloride (750) and p-Aminobenzoic acid (150); 1 mL of vitamin solution (composed by g.L^{-1}): Tyrosine (0.74), Isoleucine (1.73), Aspartic acid (4.11), Glutamic acid (4.4), Arginine (1.80), Leucine (3.37), 1-threonine (0.16), glycine (3.18), alanine (2.93), valine (2.84), methionine (0.36), phenylalanine (1.73), serine (2.2), histidine (0.66) and lysine (1.18); and 10 mL of trace element solution (composed by mg.L^{-1}): EDTA Disodium Salt (5.05), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1.52), MnCl_2 (0.34), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.10), CuSO_4 (0.10), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.13), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.52), FeSO_4 (1.01), Boric Acid (0.34) and KI (0.03). The inoculum concentration was fixed according to the experimental design described in the item experimental design for mixing (Table 1). The fermentations were conducted in duplicate at 25 °C, initial pH of 5.2 and 150 rpm, during 120 hours with withdrawals of samples at the times 0, 15, 24, 48, 72, 96 and 120 hours.

2.3 Experimental design for mixing

It was used experiment research methods of nature quantitative according Pereira et al. (2018). MONA, PE-2, CAT-1 and ATCC strains proportions were defined using the response surface methodology for mixing. The design of simplex-centroid mixtures was composed of MONA (x_1), PE-2 (x_2), CAT-1 (x_3) and ATCC (x_4) variables with the combination number equal to 2^{n-1} , which is the number of components or variables, plus the central point. The responses of this design were biomass, ethanol, residual sucrose and glycerol. Table 1 shows the real and codified levels of the studied variables. The design of the four components was composed of 15 trials.

Table 1. Experimental design of simplex-centroid mixtures 2^{n-1} plus a central point to and mean values of the response.

N° of Essay	Levels of coded and real independent variables				Response				
	MONA (g.L^{-1})	PE-2 (g.L^{-1})	CAT-1 (g.L^{-1})	ATCC (g.L^{-1})	Biomass (g.L^{-1})	Ethanol (%v/v)	Residual Sucrose (g.L^{-1})	Glycerol (g.L^{-1})	Acetic Acid (g.L^{-1})
1	1.00 (0.09)	0 (0.00)	0 (0.00)	0 (0.00)	9.84 ± 0.47	9.57 ± 0.18	25.42 ± 0.96	6.52 ± 0.57	0.29 ± 0.02
2	0 (0.00)	1.00 (0.09)	0 (0.00)	0 (0.00)	8.25 ± 0.26	10.24 ± 0.17	24.81 ± 1.29	8.42 ± 0.51	0.20 ± 0.00
3	0 (0.00)	0 (0.00)	1.00 (0.09)	0 (0.00)	8.58 ± 0.82	10.34 ± 0.30	32.90 ± 0.14	7.75 ± 0.51	0.35 ± 0.02
4	0 (0.00)	0 (0.00)	0 (0.00)	1.00 (0.09)	5.27 ± 0.35	9.37 ± 0.58	21.61 ± 3.22	7.12 ± 0.20	0.16 ± 0.01
5	0.5 (0.045)	0.5 (0.045)	0 (0.00)	0 (0.00)	9.63 ± 0.10	9.91 ± 0.17	45.38 ± 2.74	6.86 ± 0.01	0.25 ± 0.01
6	0.5 (0.045)	0 (0.00)	0.5 (0.045)	0 (0.00)	9.61 ± 0.40	9.87 ± 0.27	54.39 ± 1.73	6.34 ± 0.18	0.10 ± 0.01
7	0.5 (0.045)	0 (0.00)	0 (0.00)	0.5 (0.045)	8.44 ± 0.39	10.00 ± 0.16	25.27 ± 4.48	6.53 ± 0.48	0.25 ± 0.02
8	0 (0.00)	0.5 (0.045)	0.5 (0.045)	0 (0.00)	9.30 ± 0.06	10.89 ± 0.69	27.41 ± 0.83	7.55 ± 0.33	0.37 ± 0.01
9	0 (0.00)	0.5 (0.045)	0 (0.00)	0.5 (0.045)	8.04 ± 0.50	10.23 ± 0.18	22.66 ± 1.24	6.76 ± 0.07	0.29 ± 0.01
10	0 (0.00)	0 (0.00)	0.5 (0.045)	0.5 (0.045)	9.71 ± 0.51	9.78 ± 0.31	38.16 ± 2.31	7.87 ± 0.17	0.19 ± 0.01
11	0.33 (0.03)	0.33 (0.03)	0.33 (0.03)	0 (0.00)	8.14 ± 0.05	9.87 ± 0.52	60.00 ± 5.93	7.23 ± 0.33	0.21 ± 0.00
12	0.33 (0.03)	0.33 (0.03)	0 (0.00)	0.33 (0.03)	8.42 ± 0.49	9.89 ± 0.01	48.46 ± 1.05	6.54 ± 0.10	0.30 ± 0.00
13	0.33 (0.03)	0 (0.00)	0.33 (0.03)	0.33 (0.03)	9.00 ± 0.17	9.54 ± 0.76	68.22 ± 0.96	7.00 ± 0.06	0.19 ± 0.01
14	0 (0.00)	0.33 (0.03)	0.33 (0.03)	0.33 (0.03)	7.63 ± 0.15	9.99 ± 0.30	55.81 ± 1.36	7.18 ± 0.07	0.18 ± 0.00
15	0.25 (0.02)	0.25 (0.02)	0.25 (0.02)	0.25 (0.02)	8.10 ± 0.77	9.56 ± 0.59	57.73 ± 0.37	7.21 ± 0.17	0.18 ± 0.00

In levels, the numbers were coded variable (real value). Data presented as average ± standard deviation (n=2). Source: Authors.

The representations of the adjustment of the response values, which are ethanol, biomass, acetic acid, and residual sucrose concentration, were fitted to a linear (Equation 1), quadratic (Equation 2) and cubic (Equation 3) model in term of

MONA, PE-2, CAT-1 and ATCC yeasts. The statistical significance of the equations was made through analysis of the variance, at the level of 10% confidence.

$$\hat{y} = \sum_{i=1}^q b_i x_i \quad (1)$$

$$\hat{y} = \sum_{i=1}^q b_i x_i + \sum_{i<j}^q \sum_{i<j} b_{ij} x_i x_j \quad (2)$$

$$\hat{y} = \sum_{i=1}^q b_i x_i + \sum_{i<j}^q \sum_{i<j} b_{ij} x_i x_j + \sum_{i<j<k}^q \sum_{i<j<k} b_{ijk} x_i x_j x_k \quad (3)$$

The quality of the model adjustments to the experimental data was verified through analysis of variance (ANOVA) for model adjust selected of the data, and commonly used in modelling, it is necessary to check firstly if the regression is significant and if there is evidence of lack of adjustment (Breitkreitz et al., 2014). The ANOVA was performed for the models, at the level of 10% of significance and the dates analyses was used F test for regression and lack of fit and compared with $F_{\text{tabulated}}$. It has been judged model with significant regression in described experiment results that what the $F_{\text{calculated}}$ were higher $F_{\text{tabulated}}$ and p value p were next zero and models performed lack of fit that $F_{\text{calculated}}$ were higher $F_{\text{tabulated}}$ and value p were nest zero, in other words, the lack of fit were significant (Table 1 suplementar).

2.4 Analytical determinations

The biomass growth was quantified by the optic density at 600 nm and converted to dry biomass X (g.L⁻¹) through the equation that relates them ($X = 0,3693 * DO_{600nm} + 0,0217$).

The supernatants were analyzed by CLAE. In a Thermo chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with an ultraviolet refractive index detector. The column used was HyperREZTM XP Carbohydrate H+8 μm (Thermo Fisher Scientific), which was protected by HyperREZTM XP Carbohydrates (Thermo Fisher Scientific). The conditions used in the analysis were 1,5 mM eluent of H₂SO₄; flow rate of 0,6 mL.min⁻¹ and temperature of 50 °C. The samples were diluted five times, filtered on a 0,45 micron nylon filter (Symta, Madrid, Spain) and analyzed in duplicate. Ethanol, sucrose, glycerol and acid acetic were analyzed in the times 0, 15, 24, 48, 72, 96 and 120 hours.

2.5 Treatment of the results

The results were first evaluated through statistical analysis using software R, version 2.0.0 (Core Team), followed by multivariate variance analyzes by clustering using the cluster methodology by the Ward method, in which the similarity measure used to gather clusters was calculated as the sum of squares between the two clusters made on all variables (Hair et al., 2009; Seidel et al., 2008).

3. Results and Discussion

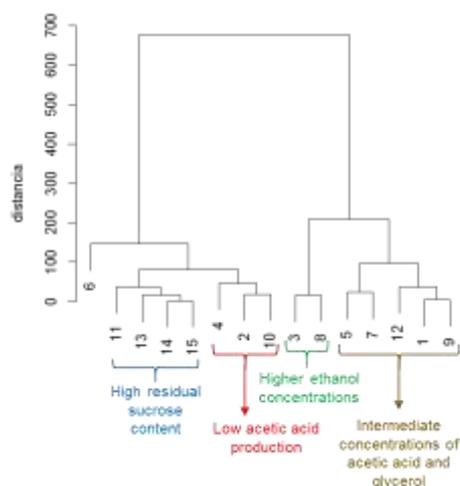
The results analyses were made in three sections. The first section was the cluster analysis used to classify elements into groups, in a way that elements within the same cluster are very similar, and elements in different clusters are different from each other. The data used were of the results of the mixtures design. The second section has reported the interaction between yeast and culture performance through analyses of kinetics parameters. It was the evaluated effects of synergic and antagonistic of the interactions between the biomass population second Amorim et al. (2011) as well as fermentative

efficiency, $Y_{P/S}$ and ethanol productivity. Lastly, it was made an assessment of the interaction between yeast through analyses of equation coefficients obtained by mixture planning. These coefficients were able to infer positive or negative effects for the different responses, such effects show how yeast metabolism responded to the interactions in co-culture.

3.1 Analysis of the cluster

In the analysis of the results of the mixtures, some tests presented similar behaviors, which indicates the existence of clusters of tests with similar performance. To validate a similar performance the cluster analysis was used, as shown in Figure 1. It is possible to observe the formation of four clusters. The tests 11, 13, 14, and 15 (combinations with three and four yeasts) form the first cluster. It was observed in the tests similar ethanol concentrations, high residual sucrose content, intermediate glycerol concentration and low acetic acid concentration (Table 1). The second group composed of the tests 4, 2 and 10, is characterized by the low acetic acid production. The third cluster was formed by the tests 8 and 3, and it was observed higher ethanol concentrations compared to the others, low residual sucrose content, intermediate glycerol quantity and high acetic acid concentration (Table 1). In experiment 3, the CAT-1 strain used was pure, while in experiment 8 it was the mixture of CAT-1 and PE-2. These two experiments are very similar since they are yeasts with similar fermentative capacity. In the fourth cluster, the tests 1, 5, 7, 9 and 12, showed intermediate concentrations of acetic acid and glycerol. In the last group, the experiment 6 was isolated, characterized by the lower acetic acid and glycerol concentration, being the combination of MONA and CAT-1.

Figure 1. Cluster Dendrogram of the clusters referring to the responses of biomass, ethanol, residual sucrose, glycerol and acetic acid from experimental design.



Source: Authors.

From of analysis of the cluster was possible to identify that assay made with three yeast was that had a greater amount of residual sucrose, whereas assay with one and two yeast, CAT-1 or PE-2 was that presented higher ethanol concentration.

3.2 Analysis of the kinetics parameters

Table 2 is presented the results of the kinetics parameters of the experimental design about substrate conversion factor into the biomass ($Y_{X/S}$), substrate conversion factor in the product ($Y_{P/S}$), ethanol yield and fermentation efficiency. In addition to showing data of the final concentration of biomass, ethanol, and sucrose (mean \pm standard deviation).

Table 2. Kinetic parameters of the mixing design tests MONA, PE-2, CAT-1 and ATCC for 120 hours.

	Biomass		Ethanol			Fermentation efficiency (%)	Sucrose
	Final (g.L ⁻¹)	$Y_{X/S}$ (g.g ⁻¹)	Final (% v/v)	$Y_{P/S}$ (g.g ⁻¹)	Yield (g.L ⁻¹ .h ⁻¹)		Final (%)
Essay 1	9.84 \pm 0.47a	0.053 \pm 0.003a	9.57 \pm 0.18a	0.409 \pm 0.010a	0.629 \pm 0.012a	76.13% \pm 1.86a	12.1% \pm 0.46b
Essay 2	8.25 \pm 0.26a	0.044 \pm 0.002a	10.24 \pm 0.17a	0.436 \pm 0.010a	0.673 \pm 0.011a	81.20% \pm 1.91a	11.81% \pm 0.61b
Essay 3	8.58 \pm 0.82a	0.048 \pm 0.005a	10.34 \pm 0.30a	0.461 \pm 0.014a	0.680 \pm 0.020a	85.74% \pm 2.53a	15.67% \pm 0.07a
Essay 4	5.27 \pm 0.35b	0.027 \pm 0.002b	9.37 \pm 0.58a	0.392 \pm 0.031a	0.616 \pm 0.038a	73.04% \pm 5.77a	10.29% \pm 1.54b
Essay 5	9.63 \pm 0.10a	0.058 \pm 0.000ab	9.91 \pm 0.17a	0.475 \pm 0.016ab	0.652 \pm 0.011a	88.46% \pm 2.99ab	21.61% \pm 1.31ab
Essay 6	9.61 \pm 0.40a	0.061 \pm 0.003a	9.87 \pm 0.27a	0.500 \pm 0.008a	0.649 \pm 0.018a	93.14% \pm 1.5a	25.90% \pm 0.82a
Essay 7	8.44 \pm 0.39ab	0.045 \pm 0.003c	10.00 \pm 0.16a	0.427 \pm 0.004b	0.658 \pm 0.010a	79.50% \pm 0.69b	12.03% \pm 2.13c
Essay 8	9.30 \pm 0.06ab	0.050 \pm 0.000bc	10.89 \pm 0.69a	0.471 \pm 0.028ab	0.716 \pm 0.046a	87.57 \pm 5.17ab	13.05% \pm 0.4c
Essay 9	8.04 \pm 0.50b	0.042 \pm 0.003c	10.23 \pm 0.18a	0.431 \pm 0.005b	0.673 \pm 0.012a	80.19 \pm 0.91b	10.79% \pm 0.59c
Essay 10	9.71 \pm 0.51a	0.056 \pm 0.004ab	9.78 \pm 0.31a	0.449 \pm 0.008ab	0.643 \pm 0.020a	83.57 \pm 1.54ab	18.18% \pm 1.10b
Essay 11	8.14 \pm 0.05a	0.054 \pm 0.002ab	9.87 \pm 0.52a	0.519 \pm 0.007a	0.649 \pm 0.034a	96.60% \pm 1.31a	28.56% \pm 2.82ab
Essay 12	8.42 \pm 0.49a	0.052 \pm 0.003ab	9.89 \pm 0.01a	0.483 \pm 0.004a	0.650 \pm 0.001a	89.91% \pm 0.71a	23.08% \pm 0.50c
Essay 13	9.00 \pm 0.17a	0.063 \pm 0.002a	9.54 \pm 0.76a	0.531 \pm 0.039a	0.627 \pm 0.050a	98.78% \pm 7.24a	32.49% \pm 0.46a
Essay 14	7.63 \pm 0.15a	0.049 \pm 0.001b	9.99 \pm 0.30a	0.511 \pm 0.011a	0.657 \pm 0.020a	95.13% \pm 1.99a	26.58% \pm 0.65bc
Essay 15	8.10 \pm 0.77a	0.053 \pm 0.005ab	9.56 \pm 0.59a	0.495 \pm 0.032a	0.629 \pm 0.039a	92.20% \pm 5.95a	27.49% \pm 0.18abc

Source: Authors.

The assay with the combination of MONA/CAT-1 (Assay 8) deserves mention due to its fermentative characteristics (Table 2). This combination presented neither the highest amount of biomass and or the highest amount of final ethanol, but it was the one that reached the highest conversion factor, $Y_{P/S}$, and the highest fermentation efficiency. This unique behavior occurred because the combination consumed the lowest concentration of sucrose, however, the sucrose consumed was probably destined from the production of biomass to ethanol production since it presented the lowest synthesis of glycerol and acetic acid. As for pure cultures, this combination was able to adapt better, because there was an increase in biomass with less substrate use and less synthesis of glycerol.

For the mixture between PE-2 yeast and ATCC (Assay 9), the lowest biomass production was verified, with lower biomass substrate conversion factor. Part of the untreated substrate in biomass was possibly destined for the production of ethanol, since the final ethanol concentration, $Y_{P/S}$, and ethanol productivity were intermediate. The fermentation efficiency of this test was the smallest.

The assay composed of PE-2 plus CAT-1 (Assay 8) showed the highest ethanol synthesis. The reason why this combination has such quality is related to the fermentative characteristics acquired in the Brazilian distilleries by the yeasts. These two strains have been reported to have a higher vigor in the metabolism of sucrose to ethanol, which environmental conditions with high concentrations of ethanol, acids and compete aggressively with savage yeasts (Basso et al., 2008; Batistote et al., 2010; Santos et al., 2017; Silva et al., 2020). This combination showed an amount of intermediate biomass and the highest concentration of final ethanol, probably the sucrose was diverted to ethanol production. Affirmative can be

confirmed by high the results of fermentative efficiency and productivity (Table 2). With respect to the pure cultures of this co-culture, this mixture was able to adapt better, as both yeasts obtained biomass gain, reaching the highest concentration of ethanol and acetic acid (Table 1 and 2).

The mixture of PE-2/CAT-1/ATCC (Assay 14) showed evidence that the biomass production route was diverted to ethanol production, since it was the co-crop that produced the least biomass and produced the most ethanol, with substrate conversion factor in lower biomass and high fermentation efficiency and productivity for ethanol production, when compared to other tests. It was also observed that this interaction promoted the lower production of acetic acid and the concentration of intermediate glycerol.

The MONA/CAT-1/ATCC combination (Assay 13) presented distinct fermentation characteristics, as it produced the highest concentration of final biomass and the lowest concentration of ethanol, evidencing the deviation of the metabolic route of ethanol for the synthesis of biomass. However, it reached the highest fermentation efficiency (98.78%), due to the lower consumption of sucrose. Even with the highest biomass production, the mixture did not divert the sugar to glycerol production, nor did it produce the highest concentration of acetic acid to meet the demand for biomass synthesis (Rodrigues et al., 2005).

Trials with three and four yeasts, in general, presented favorable kinetic behavior for ethanol production on an industrial scale, since the biomass produced was lower, with lower consumption of sucrose and less production of undesirable metabolites (acetic acid and glycerol). Although they did not reach equal or higher ethanol concentration of CAT-1/PE-2, it was the co-cultures that presented the highest fermentative efficiency. In view of this, the combinations with three yeasts showed relevant characteristics that should be deepened in future studies.

3.3 Analysis of the mathematic models

The mathematic models follow describe the production of biomass, ethanol and glycerol and consumption of sucrose. All models performed had significant regression to experiment results, but the model for residual sucrose had a lack of fit. However, all models had a reasonable predictive capacity with much information about interactions between yeast in level biological.

$$\text{Biomass (g.L}^{-1}\text{)} = 10.25x_1 + 8.39x_2 + 8.63x_3 + 5.51x_4 + 3.12x_2x_3 + 4.21x_2x_4 + 10.11x_3x_4 - 33.97x_1x_2x_3 - 47.88x_2x_3x_4 \quad (4)$$

$$\text{Ethanol (\%v/v)} = 9.54x_1 + 10.34x_2 + 10.27x_3 + 9.42x_4 + 1.9x_1x_4 + 1.89x_2x_3 - 12.45x_1x_2x_3 - 13.10x_1x_2x_4 \quad (5)$$

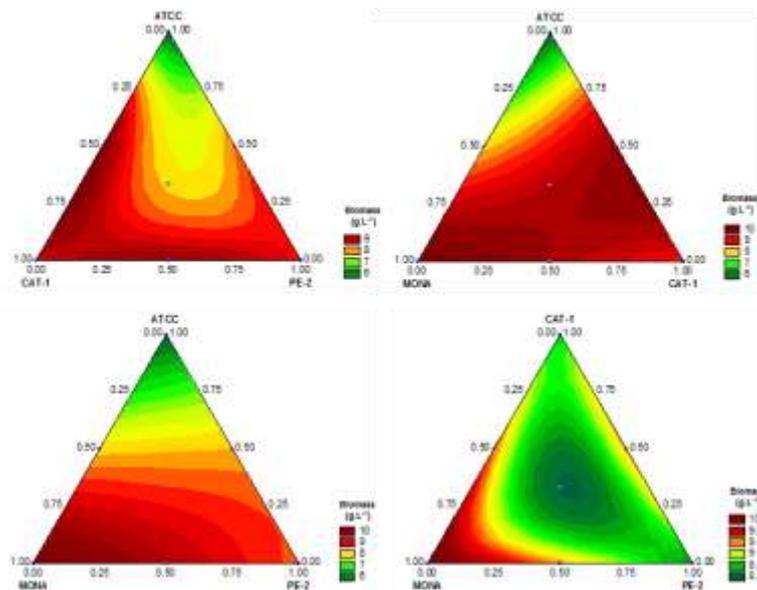
$$\text{Residual sucrose (g.L}^{-1}\text{)} = 26.26x_1 + 24.65x_2 + 32.68x_3 + 22.53x_4 + 84x_1x_2 + 104x_1x_3 + 46.55x_3x_4 + 205.28x_1x_2x_3 + 297.05x_1x_2x_4 + 558.74x_1x_3x_4 + 550.1x_2x_3x_4 \quad (6)$$

$$\text{Glycerol (g.L}^{-1}\text{)} = 6.43x_1 + 8.42x_2 + 7.74x_3 + 7.02x_4 - 2.32x_1x_2 - 2.89x_1x_3 - 2.18x_2x_3 - 3.91x_2x_4 + 2.05x_3x_4 + 15.68x_1x_3x_3 \quad (7)$$

By the equation referring to biomass in addition to all yeasts presented a positive effect the interaction between x_2x_3 , x_2x_4 and x_3x_4 were also positives characterizing synergisms between CAT-1/PE-2, CAT-1/ATCC, and PE-2/ATCC. This same conclusion can be seen in the biomass level curves (Figure 2) where the red region represents the highest concentration of biomass and the green region the lowest concentration of biomass. Analytically, CAT-1 and PE-2 (x_2x_3) yeasts have high fermenting power and robust biochemical machinery to withstand osmotic stress and tolerate high ethanolic concentration,

these factors contributed to evidence a synergistic effect between CAT-1 and PE-2. However, when the yeast MONA or ATCC was added, the mixtures showed the lowest biomass production, maybe justification has been the high competitive strength among yeasts that led to behavior the antagonistic effect in co-culture (Figure 2, Table 1).

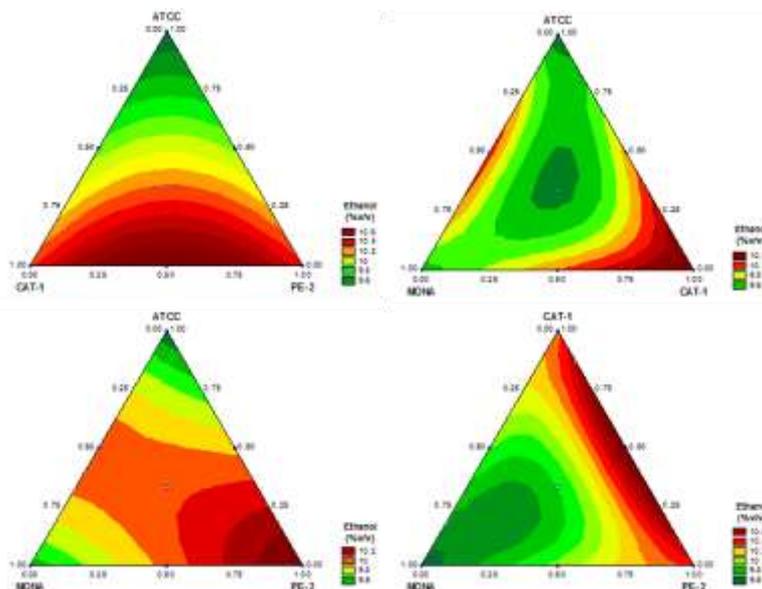
Figure 2. Contour regions of the biomass concentration response for the quaternary combination used in the experimental design for simplex centroid mixtures.



Source: Authors.

It is important to highlight that MONA yeast has been isolated from grapes Monastrell, but presented metabolic characteristics adapted and robust to the sugar cane environment due to it presents the highest concentrations of biomass and ethanol (Table 1, Figure 2 and Figure 3).

Figure 3. Contour regions of the ethanol concentration response for the quaternary combination used in the experimental design for simplex centroid mixtures.

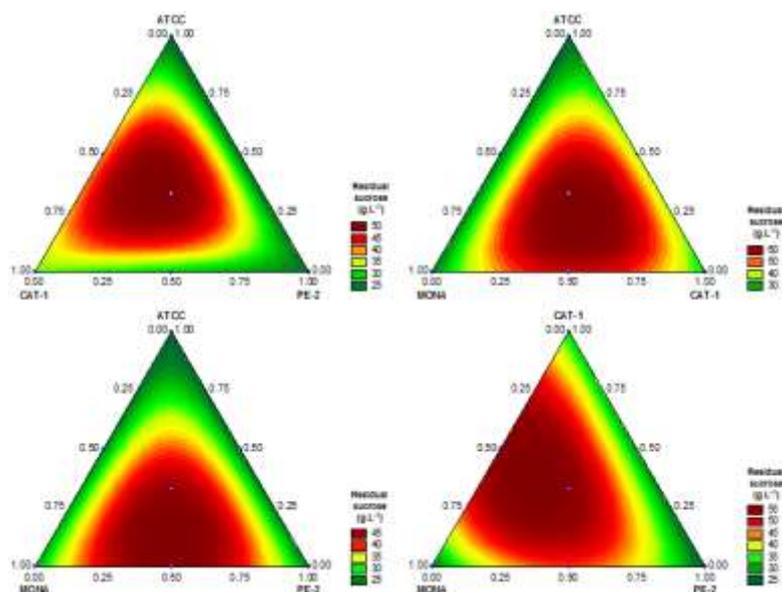


Source: Authors.

Analyzing the equation that describes ethanol production, it was possible to observe that in addition to all yeasts showed a positive effect, the interaction between x_1x_4 and x_2x_3 was also positive, characterizing synergism between MONA/ATCC and CAT-1/PE-2. In the level curves for ethanol (Figure 3), CAT-1 and PE-2 (x_2x_3) yeasts always stand out because they are in the region with the highest concentration of ethanol and thus presented a synergistic effect when cultivated together. As previously discussed, these yeasts are of industry ethanol from sugar cane (Basso et al., 2008). Already the antagonistic effect was evidenced by the coefficients of the interactions between MONA/CAT-1/PE-2 and MONA/PE-2/ATCC.

Analyzing the model for residual sucrose, it was possible to observe that in addition to all yeasts showed a positive effect the interaction between two yeasts and three yeasts also obtained the same effect (Table 3). In terms of coefficients, the interactions with three yeasts are likely to leave more sucrose in the medium than the others as seen in the residual sucrose level curves (Figure 4).

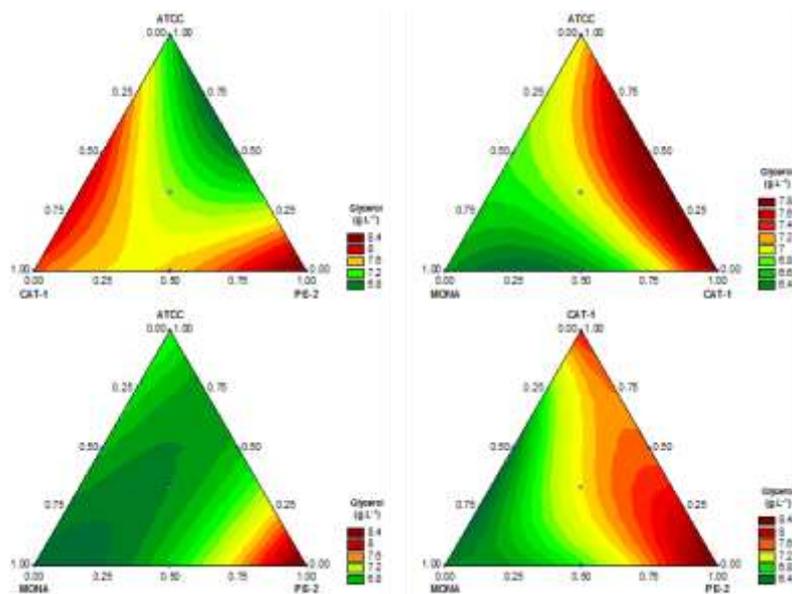
Figure 4. Contour regions of the residual sucrose concentration response for the quaternary combination used in the experimental design for simplex centroid mixtures.



Source: Authors.

Through the mathematical model for the glycerol response, it was possible to observe that the linear coefficients, as well as the tertiary coefficients, had a positive effect, therefore when the yeasts are cultivated in a mixture with three propitiates the production of glycerol. The interactions between two yeasts (quadratic coefficients) were mostly negative, evidencing the decrease of glycerol production. In Figure 5 it was noted that MONA is frequently present in the region of lower concentration of glycerol, whereas CAT-1 and PE-2 yeasts were more frequent in the higher concentration region. The fact that CAT-1 and PE-2 produce a higher amount of glycerol is corroborated by the higher production of ethanol since glycerol is part of the ethanol adaptation route (Rodrigues et al., 2005). PE-2 yeast produced the highest concentration of glycerol in pure culture (Table 1). In the studies of Santos et al. (Santos et al., 2017), it has been reported that PE-2 yeast is less robust than the CAT-1 strain, since CAT-1 has abundant proteins involved in oxidative stress response (Sod1 and Trx1) and trehalose synthesis (Tps3), therefore it is common for CAT-1 to produce less glycerol compared to PE-2.

Figure 5. Contour regions of the glycerol concentration response for the quaternary combination used in the experimental design for simplex centroid mixtures.



Source: Authors.

4. Conclusion

In conclusion, the effect of interaction between yeast *Saccharomyces cerevisiae* CAT-1, PE-2, ATCC, and MONA was noticed through the variation of final concentration ethanol, biomass, residual sucrose, and glycerol, coefficients of models, as well as in analyzed kinetic parameters. Thus yeasts that are not often associated with sugar cane fermentation (ATCC and MONA) when in the presence of CAT-1 and PE-2 led to the antagonistic feat decreasing the biomass concentration. While the mixture with CAT-1 and PE-2 were the yeasts that strongly favored ethanol production with high consumption of the substrate. Already the interactions with three yeasts left more sucrose in the middle than the others. It stands out yeast MONA due to having lower glycerol concentration, character desirable in the sugar and alcohol industry. Thus, it is clear that from the statistical analysis employed it was possible to infer about the kinetic behavior of the yeasts in pure cultures as well as the effect of the interaction between them during the cultivation, data that can be applied directly in the industry for the choice of inoculum. The results, from this research one, can contribute to elaborate a strategic plan that involves molecular biology, system biology, and mixing planning for the deepening of the interactions between the yeasts during the recycling of cells in future research.

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