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Article

Optimization of the ellagic acid synthesis process at the bioreactor level using non-conventional yeasts

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Ellagic acid (EA) is a phenolic biomolecule. For its biosynthesis, a source of ellagitannins is required, such as strawberries and yeasts, as precursors of the tannase and β-glucosidase enzymes responsible for hydrolysis of ellagitannins. Two experimental mixture designs were applied., varying the yeast concentration and the number of ellagitannins in the culture medium, evaluating the enzymatic activity and ellagic acid biosynthesis. Aiming to find the optimal compositions of the non-conventional yeasts assessed in the research to biosynthesize ellagic acid feasibly and efficiently using a response surface performing the statistical analysis in the StatGraphics® program for obtaining a higher yield and optimizing the ellagic acid synthesis process, the results indicate that the strains *Candida parapsilosis* ITM LB33 and *Debaryomyces hansenii* ISA 1510 have a positive effect on the synthesis of ellagic acid, since as its concentration increases in the mixture the concentration of ellagic acid in the medium also increases; on the other hand, the addition of *Candida utilis* ITM LB02 causes a negative effect, resulting in the compositions of 0.516876, 0.483124 and $2.58687E^{-9}$ respectively, for a treatment under the same conditions, an optimal value of ellagic acid production would be obtained. With an approximate value of 7.33036 mg/mL.

Keywords: Ellagic acid. Yeasts. Bioreactor. Optimization.

INTRODUCTION

Ellagic acid is a stable molecule with a melting point of 362 ºC, and due to its phenolic nature, it tends to react with molecules such as proteins, alkaloids, and polysaccharides (Cruz-Antonio *et al*., 2010)., it is a protein molecule present in a free in some plant species due to their metabolism (Ascacio-Valdés *et al.*, 2013), coming from the degradation of ellagitannins, which are a subgroup that belongs to tannins, polyphenolic compounds that can be obtained from the secondary metabolism of vegetables have in their structure a HHDP group (6'6 dicarbonyl 3'3, 4'4, 5'5-hexahydroxydiphenic acid or hexahydroxydiphenic acid), which is characteristic of these compounds, when ellagitannin is in the presence of acids or strong bases, hydrolyzes and releases the HHDP group, which undergoes a spontaneous lactonization reaction, forms cyclic units that give rise to the molecule known as ellagic acid (Cruz-Antonio *et al*., 2010), the molecule is for made up of two lactones (which are intermolecular esterifications), four hydroxyl groups that give it its antioxidant capacity, and two aromatic rings.

For the biosynthesis of ellagic acid, fermentation is carried out using ground strawberries and homogenized in the medium as a source of ellagitannins (Aaby *et al*., 2012) in a state of maturity of less than 50%; this is because the degree of maturity of the fruit influences the concentration of ellagitannins in the fruit., however, there is no relationship between the type of fruit and the state of maturity, since the amount of ellagitannins

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changes depending on the fruit. The skin has the highest ellagic acid content in ripe and immature fruits (Lee, Talcott, 2004). In strawberries, there is a decrease in ellagic acid as the fruit matures (Williner, Pirovani, Guemes, 2003). Yeasts were used as precursors of the extracellular enzyme's tannin acyl hydrolase or tannase and β-glucosidase, which are known to be capable of hydrolyzing the β-1,4 bonds of the ellagitannin molecule that holds the HHDP groups attached to the glucose molecule to obtain ellagic acid (De la Cruz *et al*., 2011).

The importance of obtaining ellagic acid from the degradation of ellagitannins present in strawberries, such as strawberry, lies in its effects on human health since it acts as an antioxidant agent that can prevent the adverse effects of reactive species on the functions and physiological factors of the body, such as free radicals that give rise to degenerative diseases (Han, Lee, Kim, 2006).

The present work evaluates in a semi-qualitative and semi-quantitative way the capacity of 7 non-conventional yeast strains, *D. hansenii* PYC 2968, *D. hansenii* ISA 1510, *C. utilis* ITM LB02, *C. parapsilosis* ITM LB33, *Pichia pastoris* PYC GS115, *Pichia kluyveri* Y13 and *Issatchenkia terricola* Y14 for the enzymatic production ability of tannin acyl hydrolase and β-glucosidase to degrade ellagitannins individually and in consortia to biosynthesize ellagic acid. The study was divided into four stages; the first one focused on the evaluation of the individual enzymatic activity in a plaque of each of the seven non-conventional yeasts, the second in the assessment of microbial consortia to identify the strains that can coexist together without showing antagonistic behavior and thus select which microorganisms can be used in double or triple cultures depending on the number of strains that can interact together without inhibition between them, the third focused on the evaluation of the development of the selected consortia in the competition level tests. Flask took as the main evaluation criterion the amount of ellagic acid synthesized in the entire kinetics course. The last stage focused on finding the optimal yeast compositions to biosynthesize ellagic acid aiming to obtain the optimal production points in the process using response surface using the program StatGraphics®.

MATERIAL AND METHODS

Non-conventional yeast strains

The non-conventional yeast strains used in this work are obtained from isolates of spontaneous fermentations in the production of Mezcal with ITM LB nomenclature; others are strains obtained from the strain of the Laboratorio de Biotecnología of the Instituto Tecnológico Superior de Ciudad Hidalgo with Y nomenclature, others from Portugal Yeast Culture with PYC nomenclature, from the Instituto Superior de Agronomía with ISA nomenclature. The strains used in the present work were *D. hansenii* PYC 2968, *D. hansenii* ISA 1510, *C. utilis* ITM LB02, *C. parapsilosis* ITM LB33, *P. kluyveri* Y13, *I. terricola* Y14, and *P. pastoris* PYC GS115, which were maintained and conserved in the stock of the Laboratorio de Bioquímica of the Instituto Tecnológico de Morelia.

Obtaining and preserving the strawberry

The strawberries used in the fermentations were obtained from the municipality of Lagunillas Michoacán, Mexico, Coordinates 19°34′15 ″ N 101°25′10″ W. These were cut at a stage lower than 50% of maturation and washed. The end and leaf were removed and stored in deep freezing at -80 °C (Revco Panasonic, Model MDF-U5486SC-PA). Before fermentation, they were thawed and ground in a food processor for easy incorporation into the bioreactor medium.

Conditions of cultivation and growth of yeasts in a flask

A YPD medium composed of $MgSO_4$ (0.5 g/L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), Na_2HPO_4 (3 g/L), $CaCl_2$ (0.02 g/L), casein peptone (10 g/L), yeast extract (10 g/L) and glucose (20 g/L) was prepared with strawberry under conditions of 30 ºC, 24 hours and 180 rpm in shaker SI-300R. $3x10^6$ cells/mL were inoculated using a Neubauer LUZERNE camera, 0.0025 mm Tiefe Depth model for the inoculum volume, in addition to each of the kinetics, five samples of 1 mL were taken separately, making measurements every 4 hours, the overeating was

centrifuged and separated to store in the refrigerator at -3 ºC which was used for enzymatic testing, sugar determination, and ellagic acid determination (Taken and modified from Aguilar *et al*., 2015).

Experimental design

Table I shows the SIMPLEX-LATTICE mixture design developed by the StatGraphics® program was used, with variables corresponding to the three selected non-conventional yeasts, *C. utilis* ITM LB02, *C. parapsilosis* ITM LB33 and *D. hansenii* ISA 1510. The amount of ellagic acid determined by HPLC was analyzed as a response variable. 20 bioreactor kinetics were made, ten treatments for each experimental design, 1200 mL of strawberry for a total volume of 2000 mL for the first design (D1) consisting of the treatments 1-10 and 750 mL of strawberry for a total volume of 1500 mL for the second design (D2) consisting of the treatments 11-20, to observe the behavior of yeasts according to kinetic parameters, the composition of the experimental design of the consortia is shown

TABLE I - Experimental design SIMPLEX LATTICE obtained from the StatGraphics®

Treatment	*D. hansenii* ISA 1510	*C. utilis* ITM LB02	*C. parapsilosis* ITM LB33	Treatment	*D. hansenii* ISA 1510	*C. utilis* ITM LB02	*C. parapsilosis* ITM LB33
1	0	0.5	0.5	**11**	0	0.5	0.5
2	0.3333	0.3333	0.3333	**12**	0.3333	0.3333	0.3333
3	0	0	1	**13**	0	0	1
4	0.1667	0.1667	0.6667	**14**	0.1667	0.1667	0.6667
5	0.5	0.5	0	**15**	0.5	0.5	0
6	0.1667	0.6667	0.1667	**16**	0.1667	0.6667	0.1667
7	0.6667	0.1667	0.1667	**17**	0.6667	0.1667	0.1667
8	0.5	0	0.5	**18**	0.5	0	0.5
9	1	0	0	**19**	1	0	0
10	0	1	0	**20**	0	1	0

Culture and growth conditions of yeasts in a Batch type bioreactor

A YPD medium was prepared composed of $MgSO_4$ (0.5 g/L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), Na_2HPO_4 (3 g/L), $CaCl_2$ (0.02 g/L), casein peptone (10 g/L), yeast extract (10 g/L) and glucose (20 g/L) with the strawberry, once the bioreactor has been sterilized with their respective Applikon® brand supplements with ADI1030, 3 L stirring controller containing, 2 L for D1 and 1.5 L for D2 of the formulated medium mentioned above, in an autoclave at conditions of 121 °C for 15 min at 15 psi of pressure and constant aeration of 0.20 vvm. $6x10^6$ cells/mL were inoculated using a Neubauer LUZERNE® chamber, Tiefe Depth model of 0.0025 mm to know the volume to be inoculated in the bioreactor and the kinetics were started at 180 rpm at 30 °C for 24 h. 10 mL samples were taken in a Falcon tube every three h from the bioreactor and the pH was recorded with a potentiometer that is coupled to the bioreactor in the biological controller, in addition to each of the records, five samples of 1 mL were taken, they were centrifuged and the supernatant was separated to store it in the refrigerator at -3 °C which was used for enzymatic tests, determination of sugars and determination of ellagic acid (Taken and modified from Aguilar *et al*., 2015).

Culture and growth conditions of yeasts in a Bach-type bioreactor

A YPD medium was prepared composed of $MgSO_4$ (0.5 g/L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), Na_2HPO_4 (3 g/L), $CaCl_2$ (0.02 g/L), casein peptone (10 g/L), yeast extract (10 g/L) and glucose (20 g/L) with the strawberry, once the bioreactor has been sterilized with their respective Applikon brand supplements with ADI1030, 3 L stirring controller containing 2 L for D1 and 1.5 L for D2 of the formulated medium mentioned above, in an autoclave at conditions of 121 °C for 15 min at 15 psi of pressure and constant aeration of 0.20 vvm. $6x10^6$ cells/mL were inoculated using a Neubauer LUZERNE chamber, Tiefe Depth model of 0.0025 mm, to know the volume to be inoculated in the bioreactor, and the kinetics were started at 180 rpm at 30 °C for 24 h. 10 mL samples were taken in a Falcon tube every three h from the bioreactor and the pH was recorded with a potentiometer that is coupled to the bioreactor in the biological controller, in addition to each of the records, five samples of 1 mL were taken, were centrifuged and the supernatant was separated to store it in the refrigerator at -3 °C which was used for enzymatic tests, determination of sugars and determination of ellagic acid (Taken and modified from Aguilar *et al.,* 2015).

pH determination

The determination of pH in the microbial fermentation kinetics carried out in flasks was carried out by direct measurement in a HANNA® Instruments potentiometer; for this technique, 1 mL of the sample was taken in conical centrifuge tubes, and the previously calibrated electrode was immersed. Measurements were made every three hours during fermentation. The pH monitoring in the bioreactor's fermentations was performed in real-time using the Applikon electrode connected to the console without pH control.

Evaluation of cell growth by Neubauer chamber

The sample consisting of 100 µL of enzymatic extract obtained from growth kinetics, 890 µL of sterile water, and ten µL of methylene blue were prepared to stain non-viable cells and distinguish them from living cells and to be able to observe their physical state and count them. The microorganism count was performed in the five corners of the set, the control of the chamber, and the result expressed in cells/mL (Taken and modified from Aguilar *et al*., 2015).

Determination of β-glucosidase by p-nitrophenol release method

Samples of 100 µL of enzyme extract and 900 µL of a p-nitrophenyl substrate in buffer were prepared in addition to a control preparation with 100 µL of the initial sample and 900 µL of citrate-phosphate buffer and 900 µL of a p-nitrophenyl substrate in a buffer. It was then incubated at 40 °C for one hour, the reaction was stopped with 1 mL of Na_2CO_3, and 30 min waited. Finally, the reading is taken in the PerkinElmer spectrophotometer, Lambda 35 model, at a wavelength of 400 nm (Taken and modified from Turner *et al.,* 2002).

Determination of tannase by the Rhodamine method

Samples were prepared, 50 µL of enzyme extract, 250 µL of citrate buffer, and 450 µL of methyl gallate substrate. The control was also designed with 50 µL of enzymatic extract at hour 0 and 700 µL of citrate buffer, in addition to the blank preparation using 300 µL of Rhodamine incubating with a Felisa thermobath, model FE-375 at 30 °C for five min. Once the incubation time was over, 200 µL of KOH was added to the sample, and it was again incubated at 30 °C for five min. Furthermore, four mL of deionized water was added to the sample and incubated at 30 °C for 10 min. Finally, it is read in the spectrophotometer at a wavelength of 520 nm, and the results are recorded (Taken and modified from Sharma, Bhat, Dawra, 2000).

Determination of protein by Lowry's method

Samples were taken using 10 µL of enzyme reagent from the sample taken from the bioreactor

and 490 µL of deionized water. Also, the blank was prepared with 500 µL of deionized water in addition to the addition of 2500 µL of Lowry's reagent. Once the mixture was homogenized, it was kept for 15 min in a place where light did not fall on it; after time, 250 µL of Folin Ciocalteu reagent was diluted with deionized water with a ratio of 3:1 and left to act for 30 minutes. Finally, the data obtained from the PerkinElmer Lambda 35 spectrophotometer at 550 nm and 750 nm were recorded, analyzing the results with the calibration curve previously performed with a stock of albumin (Taken and modified from Alejandro, 2009).

Determination of the number of ellagitannins by the Pyridine method

Samples were prepared with 10 µL of bioreactor extract and five blanks with 100 µL of deionized water. 2000 µL of pyridine was added to the pieces, and placed in an ice bath for 5 min. After this time, 100 µL of concentrated HCl was added, allowing five min for the reaction to occur. The ice bath was removed, and the Felisa model FE-375 thermo-bath was placed at 30 °C for 5 min. Once this, 100 µL of sodium nitrite was added, and the reading was immediately recorded on the PerkinElmer model Lambda 35 spectrophotometer at a length of 538 nm using glass cells. Once the task was finished, the sample was returned to the tube, and a time of 36 min was allowed to pass at 30 °C. Finally, the data were taken again in the spectrophotometer at a length of 538 nm (Taken and modified from Isaza *et al.,* 2007).

Determination of the amount of reducing sugars by the 3,5 dinitrosalicylic acid method

Samples were prepared with 10 µL of bioreactor extract and 90 µL of deionized water, and one blank with 100 µL of deionized water. 100 µL of 3,5 dinitrosalicylic acid (DNS) was added to the samples, and boiled for 5 min. After this time, 3000 µL of deionized water was added to dilute the pieces; the reading was recorded in the PerkinElmer spectrophotometer, Lambda 35 model at a length of 540 nm using plastic cells. After reading, calculate the amount of reducing sugars in g/L with a calibration curve using a glucose stock of 10 g/L (Taken and modified from Miller, 1959).

Determination of the amount of ellagic acid by HPLC method (High-Performance Liquid Chromatography)

The samples were prepared with 1000µL of enzymatic extract from the bioreactor with its complements, such as the AALBORG® rotameter, the ADI 1030 biological controller and the P100 agitation controller, MODEL ADI 1032, both of the Applikon® brand, in the vials, we used a CORTECS C18 2.7 µm column for phenols with HPLC grade methanol and HPLC grade acidic water previously filtered with the microfiltration equipment as mobile phase and with a flow of 0.8 mL/min in a 0-16 minute gradient: 95% (acidic water) - 5% (methanol); 17-21 minutes: 70% (acidic water) - 30% (methanol); 22-25 minutes: 60% (acidic water) - 40% (methanol); 26-30 minutes: 95% (acidic water) - 5% (methanol). The sample temperature is 25 °C, and the column is 50 °C; verify that the column and the mobile phase lines are properly sealed. Set the lamps to ultraviolet and prepare the system in the EMPOWER® program, load the phenols method at a wavelength of 280 nm and 254 nm, the samples were inserted, and the equipment was allowed to warm up for 20 to 30 min. The samples were treatment, and the retention time of ellagic acid was verified at 19 minutes (Taken and modified from Bala *et al.,* 2006).

RESULTS AND DISCUSSION

Based on the semi-qualitative and quantitative tests of enzymatic activity on plates carried out in previous experiments related to the research, it was determined that the strains of *I. terricola* Y14, *P. kluyveri* Y13, and *C. parapsilosis* ITM LB33 are the best for the production of β-glucosidase taking as a criterion larger zones of degradation of carboxymethyl cellulose (CMC) at a concentration of 20 g/L of the medium in the plate because they have a high capacity to break β-1,4 bonds of the CMC with halos of degradation of 9.60, 9.20 and 8.67 mm respectively. The degradation tests of the

polysaccharides with the 2% Congo Red test have not been previously carried out in yeasts, but concerning bacteria and filamentous fungi, results that are between 1 and 15 mm have been obtained (Teather, Wood, 1982), so the cellulolytic activity of the yeasts evaluated is somewhere between some yeasts and filamentous fungi. It should be noted that when testing the plates, the *C. parapsilosis* ITM LB33 and *C. utilis* ITM LB02 strains had a higher growth rate than the remaining five strains, which gives them greater adaptability. To *P. pastoris* PYC GS115, *D. hansenii* ISA 1510 and *I. terricola* Y14 with growth halos of 7.26 mm, 7.12mm and 7.13 mm, respectively, as the best strains for the production of tannase because they have a high capacity to hydrolyze the bonds of tannic acid in gallic acid (30 g/L) and glucose (20 g/L) to take advantage of it as a substrate (figures not shown).

According to the microbial competence tests, the strains *D. hansenii* ISA 1510, *C. utilis* ITM LB02, *P. kluyveri* Y13, *I. terricola* Y14, and *C. parapsilosis* ITM LB33 have a higher affinity for the substrate compared to the other yeasts of the experiment having a more accelerated growth and replication in addition to the fact that they did not present competitive inhibition by the substrate and for this reason, they were selected for the four possible mixtures of yeasts evaluated in kinetics at the flask level, for which, the formation of consortia with three possible combinations was considered binaries, the first between *C. utilis* ITM LB02 and *D. hansenii* ISA 1510, the second between *C. utilis* ITM LB02 and *P. kluyveri* Y13, and the third between the *C. utilis* ITM LB02 and *C. parapsilosis* ITM LB33 strains. Furthermore, the combination of *D. hansenii* ISA 1510, with *C. parapsilosis* ITM LB33 and *C. utilis* ITM LB02, is viable for a ternary mixture of yeasts.

Preliminary flask growth kinetics

The four preliminary flask kinetics were performed to observe the behavior of the yeasts according to kinetic parameters, made up of the consortia *D. hansenii* ISA 1510, and *C. parapsilosis* ITM LB33 for preliminary kinetics 1, *C. utilis* ITM LB02, and *C. parapsilosis* ITM LB33 for preliminary kinetics 2, *C. utilis* ITM LB02, *C. parapsilosis* ITM LB33 and *D. hansenii* ISA 1510 for preliminary kinetics three and *P. kluyveri* Y13 and *I. terricola* Y14 for preliminary kinetics four.

When testing the yeast consortia in preliminary flask kinetics, it was determined that the preliminary kinetics four did not obtain a growth with a trend similar to the others, nor did they use the substrate optimally; this is observed when buying the doubling times, therefore, were discarded for fermentations at the bioreactor level, when evaluating the performance of the evaluated consortia, the yeasts to use are determined.

Ellagitannins and ellagic acid concentration

The number of ellagitannins hydrolyzed in the fermentation, as shown in Figure 1, the initial and final concentrations of ellagitannins are similar for the preliminary kinetics 1, 2, and 4, on the other hand, in the preliminary kinetics three if it has an increase of the number of ellagitannins at the end of fermentation, and statistically analyzing the difference between the amounts of ellagic acid between the initial and final hours of the kinetics is significantly greater than the others as shown in Figure 2. Can be related to the amount of HHDP groups present in the molecules of the ellagitannins present in the strawberry since they have in their structure several groups of said molecules. Hence, it is attributable that the enzymes did not fully hydrolyze the amount of the ellagitannins' HHDP groups.

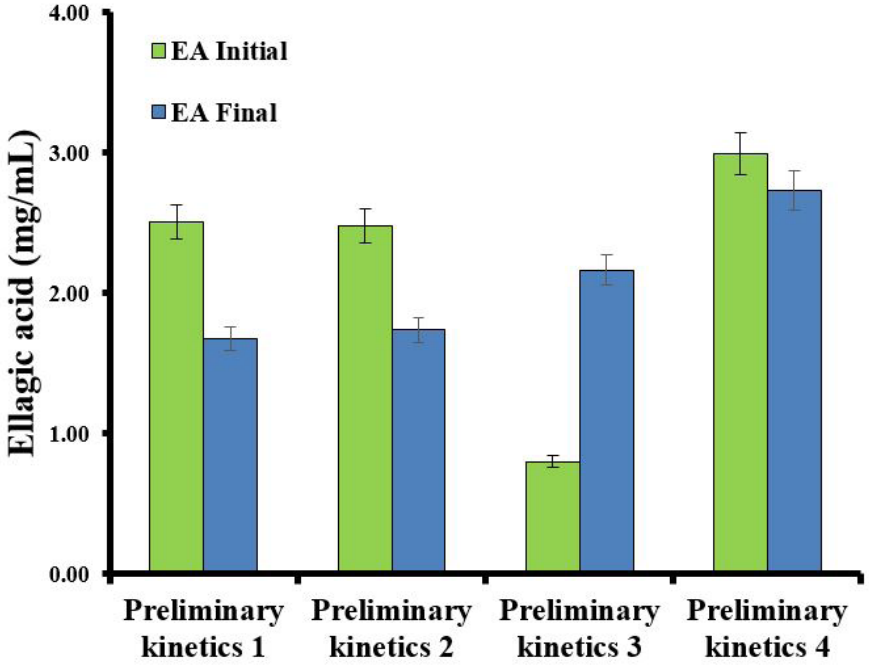

FIGURE 1 - Ellagic acid concentration (E.A.) at hours 0 (Initial) and 24 (Final) of the preliminary kinetics under conditions of 30 °C, 24 hours, and 180 rpm. Data are expressed as the mean ± standard deviation, n=2.

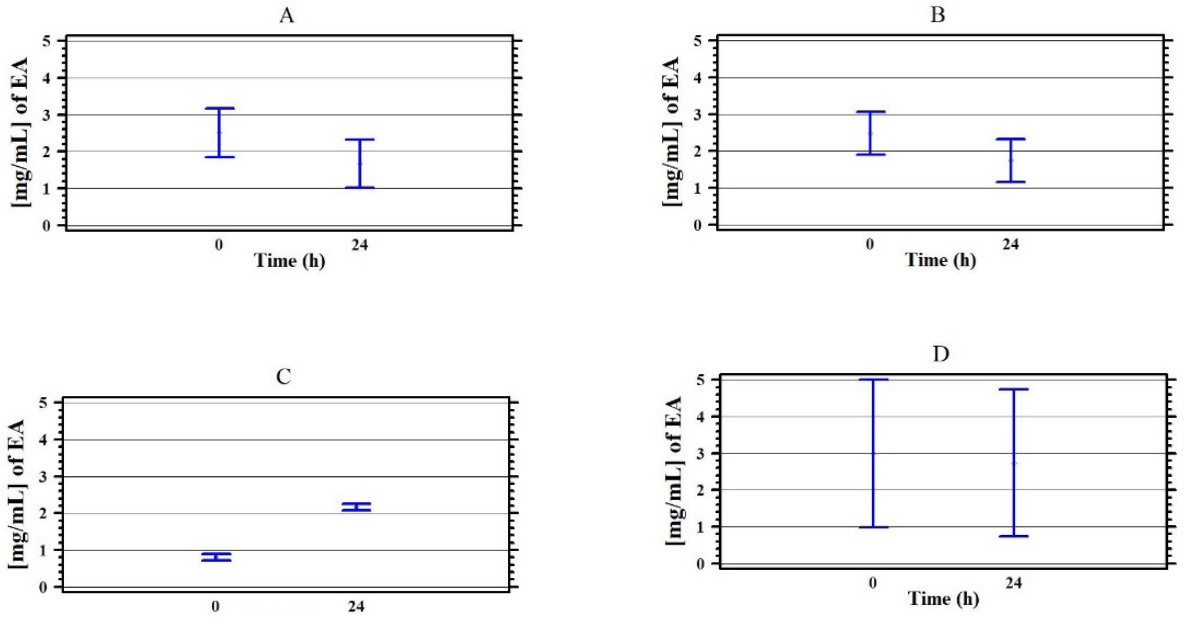

FIGURE 2 - Graph of means and 95% of Fisher LSD for the initial and final concentrations of the preliminary kinetics at 180 rpm, 30º C for 24 h. Preliminary kinetics 1 (A), preliminary kinetics 2 (B), preliminary kinetics 3 (C), preliminary kinetics 4 (D).

In the chromatographic analysis with HPLC, only the preliminary kinetics 3 shows an increase in the amount of initial ellagic acid; this may be due to different factors that involve the quality and stability of the enzymes to hydrolyze the β-1,4 bonds of the ellagitannins or to the inhibition that the yeasts might present by the synthesized ellagic acid or some by-product of the metabolism of the yeasts themselves.

Then, analyzing the results of the preliminary kinetics in the flask, the kinetics number three, by the growth rate of *C. parapsilosis* ITM LB33 and *C. utilis* ITM LB02 in CMC medium, in addition to the ability to break β 1-4 bonds of carboxymethyl- cellulose of *C. parapsilosis* ITM LB33, the ability to hydrolyze tannic acid bonds in gallic acid and glucose of the strain *D. hansenii* ISA 1510, was selected for bioreactor kinetics mainly by the concentration of ellagic acid synthesized at the end of fermentation and by the stability of the enzymatic activity of β-glucosidase, also being the only one with positive values in the yield.

Growth kinetics in Batch-type bioreactor

Cell growth

The growth kinetics carried out by a consortium of yeasts that includes: *D. hansenii* ISA 1510, *C. utilis* ITM LB02, and *C. parapsilosis* ITM LB33 carried out in the Batch type bioreactor at 180 rpm at 30 °C for 24h, in the which, the evaluation of two fermentation media was carried out, as previously mentioned, the experimental design of mixtures 1 (D1), using 1200 mL of strawberry for a total volume of 2000 mL, evaluating their cell growth with a Neubauer chamber (figures not shown).

The second experimental design of mixtures (D2) differs in that the medium consists of 750 mL of strawberry for a total volume of 1500 mL. A Neubauer chamber evaluated its cell growth (figures not shown).

There is no significant difference in the growth phases of the kinetics of the two designs, highlighting the null visibility of the lag phase in the graph because it occurs during the first hours of fermentation, which is the same time in which it is carried out at the flask level, which indicates that the yeasts adapt easily to the fermentation media. Afterward, the exponential phase of the kinetics can be observed with a duration of approximately 3 to 12 hours in the case of D1 fermentations and about 3 to 9 hours in the case of D2 fermentations. Subsequently, the beginning of the stationary phase is observed, which begins at 9 and 12 hours of fermentation, which is the process's longest stage (figures not shown).

The growth of non-conventional yeast consortia during kinetics is considered normal since it is characteristic of this type of microorganism, for which the evaluation of the kinetic parameters, such as the specific growth rate (μ), was carried out, which is a parameter indicative of the use of the substrate and the duplication time (td) that is inversely proportional to the specific growth rate. The treatments with the best performance of D1 were treatment 2 of the consortium *D. hansenii* ISA 1510, *C. utilis* ITM LB02 and *C. parapsilosis* ITM LB33 composed of 0.333 each and treatment 4 with the consortium of *D. hansenii* 1510, *C. utilis* ITM LB02 and *C. parapsilosis* ITM LB33 with a composition of 0.1667, 0.1667 and 0.6667 respectively, with a growth rate of 0.158 and 0.156 h^{-1} and duplication times of 1.89 and 1.92 hours, in the same way, the treatments with the best D2 performance were the treatment 16 of the *D. hansenii* ISA 1510 consortium, *C. utilis* ITM LB02 and *C. parapsilosis* ITM LB33 composed of 0.1667, 0.6667 and 0.1667 respectively, and treatment 17 with the consortium of *D. hansenii* ISA 1510, *C. utilis* ITM LB02 and *C. parapsilosis* ITM LB33 with a composition of 0.6667, 0.1667 and 0.1667 respectively, with a growth rate of 0.0783 and 0.0738 h^{-1} and duplication times of 1.89 and 1.92 hours, which indicates that they are the ones that make the best use of the medium substrate compared to the others treatments and that the yeast consortium and said concentration adapt so well to the fermentation medium that they have the ability to duplicate faster. (González-Hernández, Alcántar-Covarrubias, Cortés-Rojo, 2015).

Hydrogen potential

Regarding its pH behavior, the evaluated kinetics remain below the optimal range of activity of β-glucosidase which shows optimal activity at pH 5.0 (Kengen *et al.,* 1993), and tannase, pH, which shows optimal activity at 5.0 -6.0 (Rodríguez-Durán *et al.,* 2010). They have a semi-constant pH throughout the 24 hours with variations from 0.2 to 0.5 since they stabilize their environment to achieve homeostasis in the cell wall. Treatment 4, concerning the others, has a higher hydrogen potential, with values between 5.5 and 6 that are close to the optimum for

β-glucosidase, and within the range of the optimum for tannase. The treatment with the highest acidity in the medium is 10, for the others, with values around 4 and 3.5, which is due to the release of protons from organic acids to the fermentation medium or the taking of nitrogens from organic amino acids, changing to an acid character of the amphoteric that they had initially (González-Hernández, Alcántar-Covarrubias, Cortés-Rojo, 2015).

For the others, treatments 17, 18, and 20 have a higher hydrogen potential, with values around 4.6 which are the closest to the optimum for β-glucosidase and tannase. The remaining treatments obtained values around 4.4 and 4.0, which, in the same way as in D1, is due to the release of protons from organic acids to the fermentation medium or the taking of nitrogens from organic amino acids, changing to an acid character of the amphoteric they had initially (González-Hernández, Alcántar-Covarrubias, Cortés-Rojo, 2015).

The pH is a parameter that is important to keep within the range of activity of the enzymes since it helps them to remain active; this is reflected in the enzymatic activity.

Sugars reducers and ellagitannins.

The initial concentration of reducing sugars for D1 and D2 was around 20 g/L of sugars approximately; the determination throughout the fermentation was evaluated with the method of Miller (1959), and the consumption of these was carried out with speed in the two designs from the start of fermentation, the substrate decreases abruptly at the end of its exponential phase, however, although the concentration is low in the stationary phase for most of the treatments, the substrate available in the medium is not depleted, reaching the maximum consumption of sugars between hours 9 and 15 of fermentation.

On the other hand, the evaluation of the degradation of ellagitannins was carried out by the pyridine method described by Isaza *et al.* (2007). Throughout the kinetics, the amount of this compound remains constant throughout the fermentation process, indicating that the structure of the compound is very complex, and its hydrolysis is carried out only in a part of the molecule, resulting in no difference in the degradation process.

Activities Enzymatic tannase and β-glucosidase.

The tannase enzyme catalyzes the hydrolysis of tannic acid, giving as a product nine gallic acid molecules and one glucose for each substrate molecule. Based on this, a colorimetric method was used to evaluate the enzymatic activity based on quantifying the detected gallic acid described in Vázquez-Flores *et al.* (2012). For D1, treatments 7 and 9 showed high activity from the start of kinetics with maximum values of 25.01 μmol/ml*min of protein at hour 12 and 29.07 μmol/ml*min of protein at hour 18, respectively. However, they had pH values from 0.5 to 0.8 units, far from the optimum. They decreased abruptly in treatment seven at hour 24, having null tannase activity; in general, it presented an inconsistent activity during all treatments, reaching null values in some points such as It has already been mentioned, which has two possible explanations, the first is that it is because this enzyme is inducible (Doi *et al.,* 1973), requiring the presence of tannic acid in the medium so that it could be expressed, which was observed in the experiments carried out by Márquez-López *et al.* (2020), showing that by increasing the concentration of inducer the enzymatic activity also increases.

Analyzing in the same way for D2 treatments 17 and 18 showed high activity from the beginning of the kinetics, obtaining maximum values of 22.15 and 15.95 μmol/ml*min of protein at hour 12, respectively, even having pH values of 0.5 at 1.5 units away from the optimum, maintaining its activity in the course of kinetics.

On the other hand, the enzymatic activity of β-glucosidase was carried out based on the quantification of p-nitrophenyl, released by the reaction of the enzymes on the glycosidic bond of the 1-3-β-D-glucopyranoside molecule, giving favorable results for D1, treatments 2 and 10 showed high activity from the beginning of kinetics with maximum values of 13.67 μmol/ml*min of protein at hour 6 and 14.64 μmol/ml*min of protein at hour 9 respectively. However, they had pH values that were further from the optimum compared to the other runs and decreased abruptly between hours 15 and 18. For D2, treatment 11 showed high activity from the beginning of kinetics with maximum values of 15.29

μmol/ml*min of protein at hour 3. Therefore, based on the comparison of the enzymatic activities between the tannase and β-glucosidase enzymes, it was determined that the enzyme responsible for the degradation of ellagitannins was β-glucosidase.

Production of ellagic acid.

In order to compare the results with the projects carried out previously in the laboratory and recent research, they were analyzed in units of mg EA / g fruit.

Regarding the amount of ellagic acid at the end of fermentation, as shown in Table II, favorable results are obtained, the data to be compared were obtained from Ramírez-Conejo (2019) who evaluated the microbial behavior between *P. pastoris* PYC GS115, *C. utilis* ITM LB02 and *D. hansenii* ISA 1510 and the enzymatic activity for the production of ellagic acid in strawberry and blackberry by the HPLC method, which used a SIMPLEX-CENTROID design of three-factor mixtures, taking as factors each of the yeast strains varying the percentage of cell concentration of each one of them, taking as response variables the final concentration of ellagic acid and the final cell concentration, obtained values lower than 1 mg EA/g fruit in their design of mixtures, which, when making the comparison with the kinetics composed by the yeasts *C. utilis* ITM LB02, *C. parapsilosis* ITM LB33 and *D. hansenii* ISA 1510 of the present work, there are values between 3 and 5 times higher with ellagic acid concentrations in a range of 1 to 4 mg EA/g fruit for D1, with treatments 4, 7 and 10 obtaining the highest concentration, 3.25, 3.10 and 3.34 mg EA/g fruit respectively, and from 1 to 8 mg EA/g fruit, for D2 being treatments 12, 17 and 18 the ones that obtained the highest concentration, 5.64, 7.16 and 8.10 mg acid/g fruit respectively.

In turn, Ramírez-Conejo (2019) compared the enzymatic behavior of different non-conventional yeast strains for the production of ellagic acid, adding blackberry and strawberry extract to the culture medium as a source of ellagitannins, the latter being that of higher synthesis of ellagic acid registered compared to the initial concentration with a maximum of 0.78 mg of EA / g fruit. The present investigation obtains a maximum concentration of 8.10 mg EA / g fruit using a consortium of the three yeasts already mentioned, thus achieving the optimization of the process using non-conventional yeasts: however, the goal of the production of the ellagic acid greater than the current production with filamentous fungi.

TABLE II - The values obtained for ellagic acid in mg EA/fruit from Design 1, Design 2, and Ramírez-Conejo (2019).

D1		D2		Ramírez-Conejo (2019)	
Treatment	**EA**	**Treatment**	**EA**	**Treatment**	**EA**
1	2.94	**11**	3.52	**A**	0.52
2	2.86	**12**	5.64	**B**	0.16
3	2.38	**13**	4.97	**C**	0.12
4	3.25	**14**	5.07	**D**	0.11
5	1.66	**15**	5.01	**E**	0.54
6	2.17	**16**	4.38	**F**	0.74
7	3.10	**17**	7.16	**G**	0.16
8	2.88	**18**	8.10	**H**	0.80
9	2.72	**19**	3.98	**I**	0.60
10	3.35	**20**	1.73	**J**	0.68

The comparative analysis of the results with other investigations can only be carried out with the processes in which filament fungi have been used mainly because the information regarding the use of yeasts in this process is very scarce. Vattem, and Shetty (2003), analyzed the degradation of ellagitannins present in blueberries using the enzyme β-glucosidase from the *Lentinus edodes* mushroom, obtaining a maximum of 100 µg / g of dried blueberry. Huang *et al.* (2008) used a consortium of two filamentous fungi, *A. oryzae* and *Endomyces fibuliger*, obtaining 14.87% ellagic acid. Aguilera-Carbó (2009) used *A. niger* GH1 as a microorganism that produces tannase, β-glucosidase and ellagitanase, getting 3 mg/g of powdered pomegranate peel after 24 hours of fermentation. Sepúlveda *et al*. (2017) produced through a filamentous fungus such as *A. fumigatus*, using orange residues as a source of ellagitannins, obtaining 18.68 mg / g of ellagic acid.

Comparing the results obtained with Sepúlveda *et al*. (2014) in the production of ellagic acid, they brought a maximum concentration of 21.19 mg EA / g of pomegranate peel with *Aspergillus niger* GH1 as a degrading microorganism in submerged culture. Moccia *et al.* (2019) compared the fermentation of pomegranate peel powder with *A. niger* and *S. cerevisiae*, obtaining results of 9 and 45 mg EA / g of pomegranate peel in ellagic acid concentration, concluding that not only the acid production increased with the use of yeast, but also that the extractable concentration will have better yields.

According to the analysis of experiments, it was determined that the consortium of three yeasts composed of *C. utilis* ITM LB02, *C. parapsilosis* ITM LB33, and *D. hansenii* ISA 1510 del D2 favors the synthesis process since a higher production of ellagic acid it was favored with the fermentation medium that contains less strawberry pulp and therefore a lower concentration of ellagitannins in the medium, for which a possible answer is that there is inhibition of the enzymatic activity by substrate caused by a high amount of ellagitannins present in the middle of D1.

Optimization

Optimization of the response variable, the concentration of ellagic acid, was used in mg/mL in the StatGraphics® program; this simulation in the program helps to determine the combination of experimental factors that optimizes several responses at the same time. It does maximize the 'desirability' function in a range from 0.0 to 1.0. The goal of response variable optimization is currently set as maximizing the value of the response variable. The output shows the 'desirability' function evaluated at each point in the design.

The data evaluated in the optimization were of D2; they obtained higher production and yield of ellagic acid, and the minimum and maximum response values observed in the design are 1.60 and 7.49 mg/mL of ellagic acid, respectively. Table III shows these response values as a reference for desirability, indicating that data of 1.60 mg/mL has a value of 0.0, the minimum of desirability, and 7.49 mg / mL with a value of 0.99, being the maximum of desirability.

Optimize Desirability

Table IV shows the combination of factor levels that maximizes the 'desirability' function in the indicated region. It also offers the combination of factors to which the optimum is reached; adding these factors gives us an optimal value of 1.0. The StatGraphics® program carrying out this combination of the components in a mixture design shows us that if a treatment is performed under the same conditions, an optimal value of the response variable would be obtained, in this case, the production of ellagic acid with a value approximate 7.3304 mg/mL.

TABLE III - Predicted and observed desirability of the results for D2.

Treatment	Ellagic acid (mg/mL)	Desirability	
		Preview	Observed
11	3.25808	0.23134	0.281516
12	5.21797	0.6797	0.613966
13	4.59972	0.468565	0.509094
14	4.68626	0.653391	0.523774
15	4.63455	0.547499	0.515002
16	4.04756	0.362577	0.415433
17	6.62682	0.734545	0.852945

TABLE III - Predicted and observed desirability of the results for D2.

Treatment	Ellagic acid (mg/mL)	Desirability	
		Preview	Observed
18	7.49376	0.971671	0.999999
19	3.68376	0.395866	0.353722
20	1.59846	0.0202957	0.0

TABLE IV - Optimal yeast combinations

Factor	Low	High	Optimum
***D. hansenii* ISA 1510**	0.0	1.0	0.483124
***C. utilis* ITM LB02**	0.0	1.0	2.58687E-9
***C. parapsilosis* ITM LB33**	0.0	1.0	0.516876

Figure 3 shows the estimated response surface for the optimization of the ellagic acid response variable, where it is observed that the greatest desirability is directed mainly toward the strains *C. parapsilosis* ITM LB33 and *D. hansenii* ISA 1510, which implies that the addition *of C. utilis* ITM LB02 causes a decrease in desirability, therefore, for optimal production a minimum amount of *C. utilis* ITM LB02 yeast is recommended.

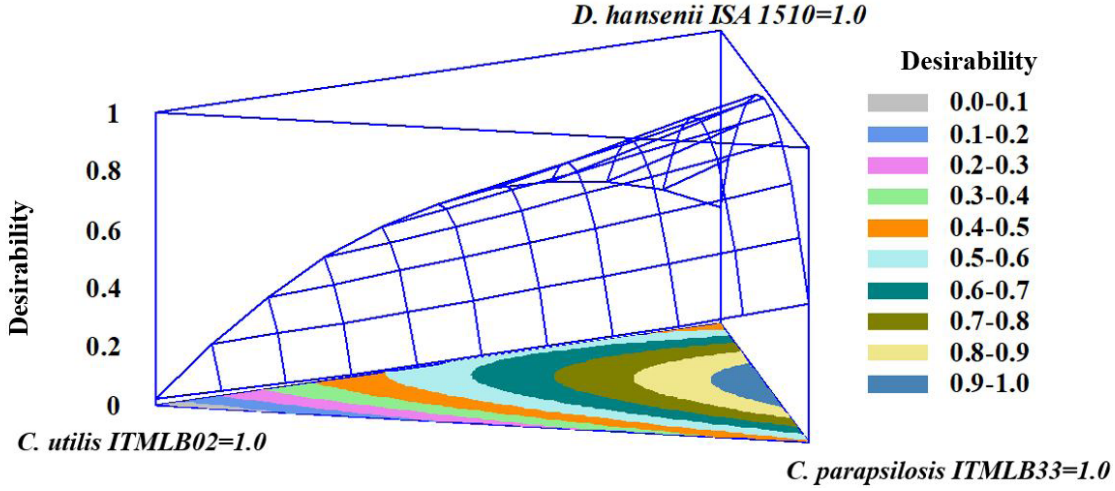

FIGURE 3 - Estimated response surface for the optimization of ellagic acid production. Quadratic model.

In general, it can be concluded that by analyzing the results obtained from the optimization of the experimental design of SIMPLEX-LATTICE mixtures in the Statgraphics® program, the proportions of the strains to conform the culture at the bioreactor level and obtain a higher yield and optimize the synthesis process of ellagic acid indicate that the proportions of the strains *C. parapsilosis* ITM LB33 and *D. hansenii* ISA 1510 have a positive effect on the synthesis of ellagic acid since as its concentration increases in the mixture, the concentration of ellagic acid also increases in the culture medium. The addition of *C. utilis* ITM LB02 causes a negative effect since as its concentration increases in the mixture, there is a decrease in the production of ellagic acid; in the conditions already evaluated, they must be mixed in fermentation to obtain an optimal ellagic acid production. The goal of ellagic acid production greater than the current production with filamentous fungi is not met.

CONCLUSION

According to the kinetics at the bioreactor level, it was observed that D2 has the appropriate conditions for greater production of ellagic acid and yield compared to the requirements of D1 and in addition to obtaining values eight times higher than the research carried out by Ramírez-Conejo (2019), being treatment 18 the one that brought the highest concentration of ellagic acid registered concerning the initial concentration using a consortium of *D. hansenii* ISA 1510 ($3x10^6$), *C. utilis* ITM LB02 (0), *C. parapsilosis* ITM LB33 ($3x10^6$), starting thus, the process of optimization of the process using non-conventional yeasts.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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