A strategy for biotechnological processes design: prickly pear (*Opuntia ficus- indica*) wine production

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For centuries, natural or spontaneous fermentation has been used to produce many types of fermented foods and beverages, playing an important socio–economic role in many civilizations. Nowadays, many of these processes continue to be applied under sub-optimal conditions, resulting in low yield and poor quality (Achi, 2005). This paper outlines the present status of prickly pear (*Opuntia ficus- indica*) wine (Mexican alcoholic beverage known as colonche) obtained by artisan fermentation of prickly pear juice, and its potential upgrade.

Prickly pear fruit (*Opuntia spp.*) is a representative icon of Mexican culture and is considered a healthy and nutritive fruit with unique sensorial characteristics. Prickly pear fruit is a juicy sweet smooth fruit with many seeds, very rich in sugars. It contains calcium, phosphorus, proteins, glucose, fructose, vitamin C, vitamin A, thiamine, riboflavin, niacin, amino acids, lipids, ascorbic acid and iron, and exhibits low acidity. In Mexico more than 393,506 ton/year are produced, mainly for unprocessed consumption as a fresh fruit in the central and northern parts of the country. Some typical products, beverages and candies, are produced from the fruit, but their commercialization is limited due to poor quality control in the manufacturing process. One of these traditional products is the so-called "Colonche", a sweet alcoholic beverage, reminiscent of the red wine, obtained by natural fermentation of juice prickly pear fruit (*O. streptacantha*) that is well appreciated for its flavor. However, the flavor from batch to batch is non-uniform, suggesting the presence of microorganisms, participating in the natural fermentation, that may lead to undesirable components depending on their capacity for metabolic adjustment to the micro environmental conditions, and adaptation to the substrate (Navarrete-Bolaños et al. 2007).

Biotechnology applied to many traditional fermentation processes can assist in upgrading them, changing the traditional process into a controllable, predictable, and efficient processes. Designing a biotechnology process requires the definition of the biological system to be used, the substrate to be transformed, and the operating conditions to be applied in order to maximize the microorganism´s metabolic activity, increase the process yield, and ensure the quality of the final product. (Achi, 2005; Navarrete-Bolaños et al, 2007). For traditional fermentations, the substrate is a natural source (for example a fruit or fruit juice) leading to a typical product (for example

matured cheese), and is seldom varied. Once the substrate is defined, one should seek the best strain or biological system to obtain the desired final product. Two alternatives are usually followed in selecting the biological system: acquiring it from a private biological resource center (for example, The American Type Culture Collection) or selecting it from natural spontaneous fermentations (Navarrete-Bolaños et al., 2007). For fermented beverages, the second option is usually the best alternative because the microorganisms involved are better adapted to perform the transformation of the specific substrate into alcohols, esters, and other aromatic compounds through a complex reaction network associated with their metabolic activity leading to the unique characteristics of aroma and flavor of the desired product. Similar results are difficult to achieve using a single purified strain.

Thus, the integral design of such a process should include guidelines for ecological studies leading to the selection of the strains more suitable for the application, as well as tools for optimizing the bioreactor operation. Activities developed for prickly pear fermented juice. The results show that a culture formed by *Saccharomyces cerevisia* and *Pichia fermentans* can be used as a starter inoculum for prickly pear juice fermentation, and that at conditions of 15°C on juice of 16°Bx containing 100 g/L of Potassium Metabisulfite allow obtain a fermented alcoholic beverage with a unique agreeable aroma and flavor.

Materials and methods

Prickly pear juice
Prickly pears (*Opuntia streptacantha*) ripened fruits from "La Tinaja" ranch located in San Diego de la Unión, Guanajuato, México., were used as a raw material. The fruits were peeled to eliminate their outer coat and the edible part was retained for our studies. The edible part was pressed and filtered to obtain a juice free of soluble solids.

Prickly pear fermented juice
Prickly pear fermented juice, known as colonche, was obtained from "la Tinaja" ranch.

Strain isolation
Samples of fermented juice were taken and inoculated on Petri dishes containing nutritive agar (Difco, Detroit, MI) for bacteria, potato dextrose agar (Difco, Detroit, MI) for yeast, and selective media formulated with prickly pear fresh juice plus bacteriological agar at 1.5% (v/w). One milliliter of fermented juice samples was spread onto the medium surface in Petri dishes and incubated at 28, 32, and 37 °C for 24, 48, and 72 h. Developed colonies with different morphologies were transferred to fresh agar mediums (nutritive, potato dextrose, and selective) and incubated again. The procedure was repeated until pure cultures were obtained (Stanier et al., 1986). In order to ensure the purity of the isolated colonies, during the whole isolation strategy and at the end of every incubation time, the colonies developed were analyzed by microscopic (model DMRAX2, Leica Microsystems GmbH, Wetzlar, Germany) methods using stain techniques and also by biochemical tests based on API Biochemical card (API CAUX, API 20E, and 20NE) (Stainer et al., 1986; Mandigan, et al., 2004).

Pure Culture Screening

The pure strains isolated, bacteria and yeast, were cultured in potato dextrose (for yeast) and nutritive (for bacteria) agar slants at 28 °C for 48 h. These conditions were considered the best conditions to favor their growth. Biomass samples containing 1×10^6 cells/mL were taken from each slant and transferred to 250 mL Erlenmeyer flasks containing 100 mL of prickly pear juice, mixed, and incubated at 28 °C and 100 rpm for 48h on a rotary shaker (model 4520, Forma Scientific, Marietta, OH) for culture screening in order to perform a partial selection strain based on capacity either for alcoholic fermentation or for production of desirable compounds associated with aroma and flavor as described below.

Experimental Strategy for strain selection

A centroid simplex experimental mixture design was applied to discriminate unnecessary strains and select only those with potential for obtaining a fermented product with attractive aroma and flavor. The design included the analysis of pure strains as well as mixed strains to evaluate synergic effects (Table 1). Each assay included in the experimental strategy was performed on the basis of the restrictions $[0<X_i<100\%$ and $X_1+ X_2 + X_3 + ... + X_p=100\%]$, were 100% correspond to 1×10^6 cells/mL inoculate on Erlenmeyer flasks containing 100 mL of prickly pear fruit juice. That is, in all assays the volume of prickly pear fruit juice on Erlenmeyer flask was 100 mL, and each flask was inoculated with 1×10^6 cells/mL. The inoculum composition was integrated by cells from a pure culture or cells from different cultures but always the inoculum contained 1×10^6 cells/mL according to Table 1 (for example, the first experimental assay must contain 1×10^6 cells/mL from the pure culture X_1, and experimental assay 6 must contain a mixture of 0.5×10^6 cells/mL from X_2 and 0.5×10^6 cells/mL from X_5). Each Erlenmeyer flask inoculated was incubated on a rotary shaker at 28 °C and 100 rpm for 48h to obtain the fermented products. The results obtained were used for the selection and discrimination of microorganisms based in the construction and solution of a third order mathematical model.

Strain Identification

Once the cultures yielding high fructose concentration were selected, strain identification was performed using molecular biology tools. In this study, the ribosomal sequence analysis of the 5.8S rRNA gene and two ribosomal internal transcribed spacers ITS1 and ITS2 were amplified by Polymerase Chain Reaction (PCR), followed by restriction analysis using endonucleases. The method is based on genomic DNA extraction from selected strains according to the protocol proposed by Ausubel et al. (1999). For rRNA gene amplification the DNA extracted was mixed with PCR super mix (Invitrogen, Carlsbad, CA) containing high-fidelity DNA polymerase (Invitrogen). The PCR products were purified with the QIAEX II kit (Qiagen, Hilden, Germany) and digested with restriction enzymes (Invitrogen). Once purified, the amplified fragment was cloned on Topo TA 2.1 (Invitrogen) and sequenced. The sequence was compared with those reported on the National Center for Biotechnology Information (NCBI) database using the "Blast" algorithm for strain identification.

Table 1 Centroid Simplex Mixture Design for 5 isolated cultures and response variable.

Assay	X_1	X_2	X_3	X_4	X_5	[a]Y_{-OH}
1	1	0	0	0	0	6.543
2	0	1	0	0	0	1.133
3	0	0	1	0	0	6.702
4	0	0	0	1	0	7.423
5	0	0	0	0	1	7.098
6	0.5	0.5	0	0	0	6.745
7	0.5	0	0.5	0	0	8.758
8	0.5	0	0	0.5	0	6.809
9	0.5	0	0	0	0.5	8.538
10	0	0.5	0.5	0	0	7.329
11	0	0.5	0	0.5	0	6.026
12	0	0.5	0	0	0.5	11.298
13	0	0	0.5	0.5	0	7.899
14	0	0	0.5	0	0.5	6.950
15	0	0	0	0.5	0.5	8.266
16	0.333	0.333	0.333	0	0	9.128
17	0.333	0.333	0	0.333	0	6.121
18	0.333	0.333	0	0	0.333	9.753
19	0.333	0	0.333	0.333	0	8.065
20	0.333	0	0.333	0	0.333	6.407
21	0.333	0	0	0.333	0.333	6.234
22	0	0.333	0.333	0.333	0	7.394
23	0	0.333	0.333	0	0.333	8.138
24	0	0.333	0	0.333	0.333	6.217
25	0	0	0.333	0.333	0.333	8.131
26	0.25	0.25	0.25	0.25	0	5.585
27	0.25	0.25	0.25	0	0.25	6.202
28	0.25	0.25	0	0.25	0.25	5.503
29	0.25	0	0.25	0.25	0.25	6.917
30	0	0.25	0.25	0.25	0.25	6.711
31	0.2	0.2	0.2	0.2	0.2	6.796

[a] Ethanol concentration (% v/v).

Experimental design for alcoholic fermentation of prickly pear juice on bioreactor
Using as a starter inoculum the strains selected, a factorial design with two centers for to quantified variables at bioreactor level was constructed (Table 2) and the experimental assays were realized. The variables considered were V_1 (sugar concentration), V_2 (Potassium Metabisulfite concentration), and V_3 (temperature) for ranges of 12-15°Bx for V_1, 100-200 mg/L for V_2 and 15-25 °C for V_3. In the experimental scheme, the variable levels are coded (-1 for the lower level, +1 for the upper level, and 0 for the mean value). Each assay was analyzed to determine alcohol yield, volatile compounds concentration based on solid-phase microextraction-gas chromatography-mass spectrometry, and sensorial analysis. The results for alcohol yield, and volatile

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compounds analysis were used for variable analysis based on mathematical model analysis.

Table 2 Factorial design for three variables and two levels (2^3) for bioreactor optimization.

Assay	V_1	V_2	V_3	[a]Y_{-OH}
1	-1	-1	1	4.0
2	0	0	0	4.5
3	1	1	1	6.0
4	-1	1	1	4.0
5	-1	-1	-1	4.4
6	1	-1	1	4.2
7	1	-1	-1	6.6
8	-1	1	-1	6.2
9	1	1	-1	4.5
10	0	0	0	4.6

[a] Ethanol concentration (% v/v).

Substrate and product fermentation analysis

Final samples of fermented juice were analyzed by a gas chromatographer with a flame ionization detector (GC/FID) PerkinElmer Claurus 500 model for ethanol quantification. Helium was used as transporter gas, hydrogen as combustible and air like FID flame starter. The analysis was made on AT-WAX 30 m x 0.25 mm ID x 0.25 μm capillary column. Also, the samples were analyzed by high performance liquid chromatography to determine sugar concentration using a modular GBC system (Scientific Equipment, Dandenong, Victoria, Australia) with refraction index detector, 75% of acetonitrile and 25% of water solution as mobile phase with a 1.20 ml/min flux, and a 250 x 4.6 mm SS Exsil AMINO 5 μm column (Grace Discovery Sciences, IL, USA). Finally, the volatile and aromatic compounds were evaluated by gas chromatography and mass spectrometry (GC/MS) Claurus 500MS (Perkin-Elmer) on a ATTM-WAX de 30 m x 0.25 mm x 0.5 μm capillary column.

Results and discussion

Microbial ecology studies. Fourteen pure cultures were isolated from prickly pear fruit fermented juice (colonche) and prickly pear fresh juice. The screening results show that only five strains were able to produce ethanol and desirable aroma. These were named X_1, X_2, X_3, X_4, and X_5, and their response studied via experimental mixture design.

Strain selection. On completion of the experiments, the results obtained (last column of Table 1) were analyzed using a combination of both descriptive and inductive statistics. In the first instance, a cubic model was constructed using least-squares to locate the strain that maximize the alcohol yield. The following expression was obtained:

Y_{OH} = 6.52X_1 + 1.104X_2 + 6.68X_3 + 7.42X_4 + 7.09X_5 + 12.80X_1X_2 + 9.36X_1X_3 - 0.28X_1X_4 + 7.20X_1X_5 + 14.63X_2X_3 + 7.57X_2X_4 + 29.23X_2X_5 + 3.57X_3X_4 + 0.34X_3X_5 + 3.77X_4X_5 - 16.25$X_1X_2X_3$ - 45.30$X_1X_2X_4$ - 30.11$X_1X_2X_5$ - 13.12$X_1X_3X_4$ - 66.10X_1X_3*X_5 -50.08$X_1X_4X_5$ - 25.44$X_2X_3X_4$ - 56.09$X_2X_3X_5$ - 94.10$X_2X_4X_5$ + 12.73$X_3X_4X_5$

The model was analyzed by statistical tools based on the analysis of variance, which suggests that the model exhibits a statistically significant relationship between ethanol concentration and the isolated cultures (variables) used. On the basis of this model the strain selection that maximized the ethanol concentration was determined by optimization techniques based on Newton-Raphson method. Results show that the independent factors that maximize the response function are $X_2 = 0.40$, and $X_5 = 0.60$, leading to a fermented product with 11.71 % of alcohol. Thus, the analytic solution suggests that the starter inoculum for the fermentation process should be formed by $4x10^5$ cells/mL of X_2 and $6x10^5$ cells/mL of X_5.

Strain identification. Once the starter inoculum was defined, the molecular strain identification was realized. The results show that strain X_2 is *Pichia fermentans*, and the strain X_5 is *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* is the yeast commonly used on alcoholic fermentations process and *Pichia fermentans* produce a wide number of aromatic compounds.

Assays on bioreactor. Once selected *Saccharomyces cerevisia* and *Pichia fermentans* as the best strains for prickly pear juice fermentation, the experimental strategy for bioreactor optimization was performed. The results, using alcohol yield as response function, show (last column of Table 2) that the operating conditions that maximize alcohol yield (6.5% (v/v)) are $V_1=16°$ Bx, $V_2=100$ mg/L of Potassium Metabisulfite concentration, and $V_3=15°C$ of temperature. These conditions also lead to the synthesis of alcohol, and aromatic and flavor related components, such as isopentyl alcohol, isopentyl alcohol acetate, ethyl octanate and phenylethyl alcohol, which together provide unique characteristics of aroma and flavor to the fermented product.

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