ETHANOL PRODUCTION BY A BEE-ISOLATED YEAST: AN EXPERIMENTAL DESIGN ANALYSIS

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ABSTRACT

Pollinator-associated yeasts are known to be naturally transferred between the animal body and the flowers they visit. In fact, while inhabiting nectaries, yeast cells ferment their sugar content and produce several volatile compounds that attract different pollinators to the flowers. Ethanol is among these volatiles. In the present study, we investigated the fermentative capacity of a bee-isolated yeast strain (*Meyerozyma caribbica* CHAP-248) through a Central Composite Design (CCD) analysis, having pH and concentrations of sucrose, peptone, and yeast extract as variables. After reaching the stationary phase of growth, media supernatants were used to determine sugar consumption and ethanol production. The so-referred strain achieved ethanol yields of up to 0.473 g/g, which correspond to an 88% fermentation efficiency, similar to what is seen in Brazilian ethanol-producing mills. The CCD analysis showed that peptone concentration has not significantly affected fermentation yield, indicating that the cells could efficiently ferment despite the nitrogen scarcity. The pH also had no effect, suggesting that this yeast can perform satisfactorily on a wide pH range. Taking into account that insect-associated yeasts are known to produce a high number of volatile compounds, the strain CHAP-248 holds promise for multiproduct biorefinery purposes.

Keywords: Sucrose. Fermentation. Yield. Productivity. Peptone.

1 INTRODUCTION

Yeasts associated to plants and insects are known to convert nectary sugars into volatile organic compounds that attract pollinators to flowers. In this triple relationship, insects are driven to a nutrient source, plants benefit from pollination, and yeasts may use the insect's body as a transport system, a new habitat (when out of flower season), or even a breeding place[1]. Nectar is especially rich in sucrose (approximately 150 g/L) and poor in amino acids. Thus, yeasts adapted to this environment are thought to be able to ferment sugars under nitrogen scarcity[2].

Although it does not usually figure among the most common attractant volatiles, ethanol is considered one of the key molecules in attracting animals to ripening fruits[3]. Regarding alcoholic fermentation processes, the most widely employed yeast is *Saccharomyces cerevisiae*. However, the current industrial strains have long been domesticated to convert sugars into ethanol[4], and they are expected not to waste carbon sources in other metabolic routes. In this sense, they may have lost genes involved in the production of other bioproducts that are interesting to different markets.

At the same time, non-conventional yeasts have recently been described to ferment sugars with high yields[5]. Thus, we hypothesized here if a bee-isolated yeast would be able to efficiently convert sucrose into ethanol and if a nitrogen source would exert significant influence on its fermentative capacity. Through an experimental design, we also investigated the effects of pH and nutrient concentration on this yeast ethanol yield and productivity capacities.

2 MATERIAL & METHODS

The yeast strain CHAP-248 was isolated from a Brazilian native bee (*Scaptotrigona postica*). The insects were drowned in synthetic YNB media (6,7 g/L of yeast nitrogen base, pH 5,0) with 10 g/L of xylose and 0.2 g/L of chloramphenicol. Flasks containing the bees were incubated at 28°C on a shaker at 145 rpm until growth was detected by turbidity. Subsequently, one loopful of each tube was streaked on plates containing the same media described above with the addition of 20 g/L of agar. Yeast morphotypes were purified by repeated streaking and isolated as described by Albarello and coworkers[6]. The strain CHAP-248 was then taxonomically identified by analyzing the variable domains D1/D2 of its large subunit (LSU) rRNA gene as we previously described[7]. The amplified DNA was concentrated, cleaned and sequenced in an ABI 3130 Genetic Analyzer automated sequencing system (Life Technologies, California, USA) using BigDye v3.1 and POP7 polymer. The sequences were assembled, edited, and aligned using the program MEGA6. Finally, the sequences obtained were compared with those included in the GenBank (https://www.ncbi.nlm.nih.gov) using its Basic Local Alignment Search Tool (BLAST).

Yeast cells were initially pre-grown on YPD solid media (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of glucose and 20 g/L of agar) during 48 h. After that, a one-microliter calibrated loopful of cells inoculated 50 mL of media with varying pH values

and different sucrose, peptone, and yeast extract concentrations, according to a Central Composite Design (CCD) matrix assembled and analyzed with the Protimiza Experimental Design software[8], as summarized in Table 1.

At six-to-ten-hour intervals, samples were harvested from the media to determine cellular growth (by optical density at 570 nm) and supernatant storage (after centrifugation at 5000 *g* for 3 min). The supernatants corresponding to the beginning of the stationary phase were filtered through 0.45 µm filters and analyzed by high-performance liquid chromatography (LCMS-2020, Shimadzu) with a refractive index detector (RID-10, Shimadzu) and a column for sugar and ethanol (Aminex HPX-87H, Bio-Rad) to determine the sucrose consumption and ethanol production. The mobile phase used 5 mM sulfuric acid at 50 °C with a 0.6 ml/min flow rate. Calibration curves were established for all samples using seven concentrations ranging from 0.25 to 20 g/L for sucrose and 0.125 to 10 g/L for ethanol.

3 RESULTS & DISCUSSION

The LSU rRNA gene of the bee-isolated yeast strain CHAP-248 exhibited 100% identity with the corresponding sequence of the *Meyerozyma caribbica* type strain CBS9966, suggesting that CHAP-248 should be classified as this species.

Meyerozyma caribbica CHAP-248 succeeded in produce high ethanol yields from sucrose within approximately 34 h incubation period (Table 1), when the cellular growth got around the stationary phase (data not shown). In the CCD's assay 10, it reached 0.473 g of ethanol per g of available sucrose, which represents an 88% fermentation efficiency (considering the stoichiometric conversion of 0.538 g/g), similar to what is seen in Brazilian ethanol-producing mills[9].

Table 1 Central Composite Design (CCD) matrix with four independent variables and two responses analyzed. Numbers between parentheses indicate the coded values. Assays 17–19 represent the central-point triplicate of the CCD.

Assay	Variables				Responses	
	pH	Sucrose (g/L)	Peptone (g/L)	Yeast Extract (g/L)	Yield (g/g)	Productivity (g/L.h)
1	3 (-1)	50 (-1)	2 (-1)	1 (-1)	0.259	0.432
2	7 (+1)	50 (-1)	2 (-1)	1 (-1)	0.295	0.366
3	3 (-1)	150 (+1)	2 (-1)	1 (-1)	0.000	0.000
4	7 (+1)	150 (+1)	2 (-1)	1 (-1)	0.063	0.326
5	3 (-1)	50 (-1)	20 (+1)	1 (-1)	0.320	0.630
6	7 (+1)	50 (-1)	20 (+1)	1 (-1)	0.327	0.653
7	3 (-1)	150 (+1)	20 (+1)	1 (-1)	0.191	0.999
8	7 (+1)	150 (+1)	20 (+1)	1 (-1)	0.180	0.900
9	3 (-1)	50 (-1)	2 (-1)	10 (+1)	0.259	0.609
10	7 (+1)	50 (-1)	2 (-1)	10 (+1)	0.473	0.556
11	3 (-1)	150 (+1)	2 (-1)	10 (+1)	0.207	1.014
12	7 (+1)	150 (+1)	2 (-1)	10 (+1)	0.234	0.992
13	3 (-1)	50 (-1)	20 (+1)	10 (+1)	0.256	0.582
14	7 (+1)	50 (-1)	20 (+1)	10 (+1)	0.346	0.619
15	3 (-1)	150 (+1)	20 (+1)	10 (+1)	0.347	1.596
16	7 (+1)	150 (+1)	20 (+1)	10 (+1)	0.320	1.491
17	5 (0)	100 (0)	11 (0)	5,5 (0)	0.344	1.008
18	5 (0)	100 (0)	11 (0)	5,5 (0)	0.356	1.127
19	5 (0)	100 (0)	11 (0)	5,5 (0)	0.288	1.080

The highest ethanol yield, though, was achieved in the lowest sucrose concentration (50 g/L), accounting for 28–36% of that found in the ethanol industry[10]. In the assays with the highest sugar concentration tested (150 g/L), the maximum ethanol yield achieved was 0.347 g/g (68% efficiency). On the other hand, it was under these conditions that yeasts reached the top ethanol productivity (Table 1, assays 15 and 16).

As expected, sucrose concentration showed a significant negative effect on ethanol yield and a significant positive effect on productivity (Figure 1), similar to what has been seen for other insect-isolated yeasts[6]. In contrast, the CCD has shown that peptone did not exert a significant effect on fermentation yield (*p*-value = 0.057), indicating that the strain CHAP-248 is able to produce elevated ethanol amounts under nitrogen restriction — as it is observed in floral nectaries, which are dwelled by yeasts that are naturally transferred between flowers and their pollinating insects[1].

Once flowers- and pollinating insects-associated yeasts are expected to produce volatile compounds other than ethanol[1], this new strain may be successfully employed in a multiproduct biorefinery context. Indeed, two of our previous studies with *M. caribbica* strains have shown that this species can also produce high amounts of ethanol from glucose and xylitol from xylose[6,11], enabling this yeast to transform first- and second-generation feedstocks.

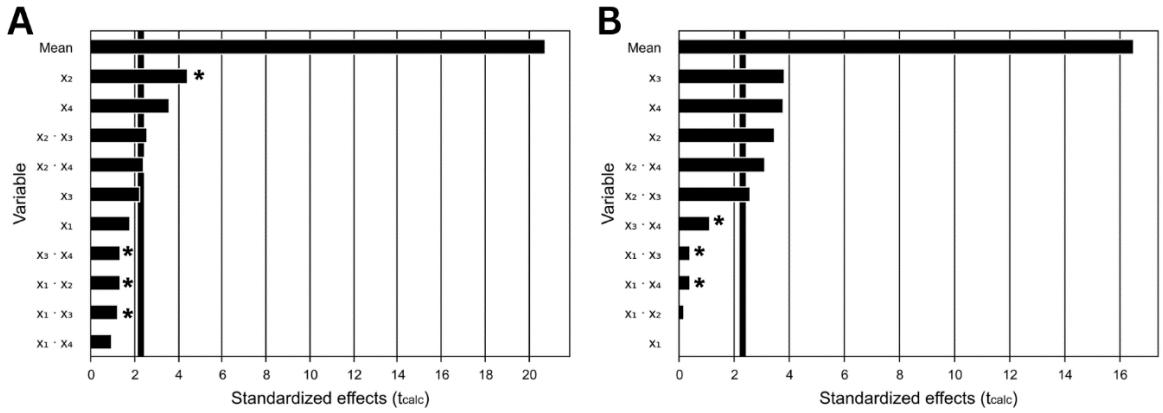

Figure 1 Pareto charts of standardized effects (calculated t) of the studied variables (x_1, pH; x_2, sucrose; x_3, peptone; x_4, yeast extract) for ethanol yield (A) and productivity (B), considering a significance level of 5%. The asterisks stand for negative calculated t (t_{calc}) values.

The pH variation also has not exerted any significant influence either in the ethanol yield (*p*-value = 0.112) or productivity (p-value = 0.960), suggesting that CHAP-248 can satisfactorily ferment sucrose in a three-to-seven pH range. It is worth noting that, in this CCD, the linear regression of experimental versus predicted values showed a high correlation coefficient (R^2 = 0.88 for both responses), indicating a satisfying correlation between them. Moreover, for ethanol yield, the $F_{regression/residuals}$ was approximately twice the tabulated *F*, and the $F_{lack\ of\ fit/pure\ error}$ approximately half of it, thus validating the following empiric coded model (Eq. 1) through ANOVA with *p*-value<0.05:

$$Y_{ethanol} = 266.61 + 24.97x_1 - 62.04x_2 + 31.15x_3 + 50.38x_4 - 18.40x_1x_2 - 17.59x_1x_3 + 13.05x_1x_4 + 35.76x_2x_3 + 33.89x_2x_4 - 18.94x_3x_4 \quad (1)$$

4 CONCLUSION

A bee-isolated yeast was shown to produce ethanol with high efficiency, with the same fermentation yield as the *Saccharomyces cerevisiae* strains employed in the Brazilian ethanol industry. Our results also indicated that the wild strain can satisfactorily ferment sucrose under nitrogen restriction and in a wide pH range. Given the high potential of pollinator-associated yeasts to produce volatile organic compounds besides ethanol, the new strain presented in this study holds promise for multiproduct biorefinery purposes. Future research could delve deep into the analysis of its volatome.

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