IMPROVEMENT OF *Actinobacillus succinogenes* THROUGH RANDOM CHEMICAL MUTAGENESIS TECHNIQUES

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ABSTRACT

Organic acids and their derivatives give rise to a lot of compounds that are widely used in industry for different applications. There is increasing interest in microbial production of this acid through the fermentative route. However, the cost of this method is still high, and to offset the petrochemical process, it is necessary to obtain more productive biological processes with higher acid yields. For this purpose, one of the natural producers of this acid is *Actinobacillus succinogenes*, which stands out for its ability to naturally metabolize a wide variety of sugars into succinic acid. Nevertheless, this production presents challenges such as product inhibition and the need for higher productivity. With this in mind, the project aimed to establish and execute a protocol for mutagenesis of *A. succinogenes* using the technique of random chemical mutagenesis to obtain clones with improved fermentative performance. The mutation was performed by exposing the strain to the mutagenic agent EMS (ethyl methanesulfonate) after conducting tests to determine the appropriate concentration and exposure time. As a result, 8 clones were obtained that showed better performance compared to the adapted strain. This demonstrates that random chemical mutagenesis by EMS was effective for the improvement of *A. succinogenes*.

Keywords: *Actinobacillus succinogenes*. Succinic acid. Chemical mutagenesis.

1 INTRODUCTION

Succinic acid is an organic acid that can be produced as the main product of anaerobic fermentation by some microorganisms[1]. This acid and its derivatives are widely used as specialty chemicals in the food, pharmaceutical, chemical and cosmetic industries[2]. The chemical industry represents the largest market for this acid[3], where it can be used in the production of biodegradable solvents, perfumes, paints and dyes, plasticizers, polyesters, and other intermediates for chemical synthesis [4]. In the food industry, succinic acid is used as a pH modifier, food preservative, and antimicrobial agent.

The production process for succinic acid can be carried out through either chemical synthesis or fermentation. Currently, the most commonly used method for commercial production is chemical synthesis, which has major disadvantages such as environmental impact due to CO2 emissions and the use of benzene, a carcinogenic compound. Therefore, there is significant interest in fermentation processes using bacteria.

Actinobacillus succinogenes has been studied with the aim of synthesizing biodegradable products, as it produces succinic acid as the main substance during fermentation, providing a natural alternative for the production of this acid[5]. Additionally, it is more tolerant to high acid concentrations and can consume a variety of carbohydrates, making it an attractive candidate for the industrial bio-production of succinic acid from various feedstocks. To achieve this, it is necessary to increase the productivity of the process to make it more economical and competitive in the market. This increase in productivity can be developed through chemical mutagenesis. Mutation is defined as any heritable change that can modify the genetic information of a given organism, potentially altering all primary components of DNA. A chemical mutagenic agent, which is more efficient due to its interactions with DNA, is used to induce this mutation [6]. EMS (ethyl methanesulfonate) is a potent mutagenic agent that induces permanent changes in DNA structure.

Considering this, the objective of the project was to define and implement a protocol to induce mutations in *Actinobacillus succinogenes* (ATCC 55618) using the random chemical mutagenesis technique, with the purpose of generating clones with higher fermentative productivity, in order to reduce the environmental impact caused by the chemical route.

2 MATERIAL & METHODS

For the development of the study, the strain *A. succinogenes* ATCC 55618 (American Type Culture Collection) was used. This strain had previously undergone adaptive evolution against increased succinic acid concentration, carried out in the 2021/2022 scientific initiation project, and was kindly provided for the chemical mutagenesis tests conducted in this work. It will be identified in this report as strain ALE7.15 (Adaptive Laboratory Evolution).

Thus, the ALE7.15 strain stored in the strain bank at -80 ºC was reactivated in TSB (trypticase soy broth) and incubated until it reached its exponential phase (optical density equal to 1.0) at 37 °C. One milliliter of this propagation culture, with the aid of a pipette, was transferred to an Eppendorf tube. The experiment was performed in duplicate. The cells were centrifuged for 5 min at 14100g and washed twice with 0.01 M sodium phosphate buffer, pH 7.0 [7]. Then, the cells were exposed to a solution of EMS 50 mg/mL (determined in a previous inactivation test) in sodium phosphate buffer at 60 mins. After,, a 5% (w/v) sodium thiosulfate solution was added in a 4:1 (v/v) ratio to the cells incubated in the EMS solution [7]. Subsequently, the cells were centrifuged again for 5 min at 14,100 g, washed twice with sterile water, and washed twice with standard culture medium (TSB).

The EMS exposure and selection strategy involved three consecutive rounds of mutagenesis, followed by the selection of microorganisms with better performance compared to the parental strain. One of the microtubes was directly plated on TSA culture, the second was plated on TSA culture with 50 g/L of succinic acid, and the third was inoculated in TSB culture for recovery in liquid culture and subsequent plating on TSA with 50 g/L of succinic acid for the selection of isolated colonies. After recovery in liquid culture and reaching an absorbance of 1 on the Bel Spectro S05, three 1 mL aliquots of each experiment were taken to continue the mutagenesis process, moving on to the second round.

After the third round chemical mutagenesis and plating, colony growth was observed on plates. These colonies are called clones because they have undergone the mutation process. To proceed, it is necessary to select them, which in this project was done randomly.

To screen the mutated strains and compare them with the adapted strain (ALE7.15) and the parental strain, fermentations were carried out in 96-well microplates. Initially, the strains were reactivated. After the growth phase, 20 µL of inoculum were transferred to each well of the microplate containing 180 µL of the culture presented in Table 2, containing 15 g/L of yeast extract. The experiments were conducted in 5 biological replicates. Incubation was performed in the Tecan Infinite 200 PRO reader at 37 °C, without agitation, for 24 h with absorbance readings at 600 nm. After the cultivation period, the cells were centrifuged at 4230g for 15 min to harvest the supernatant, which was subjected to HPLC analysis to determine sugars and organic acids. Subsequently, with the results, the maximum growth rate and the "c" coefficient were calculated to determine the performance of the mutants relative to the native strain, based on Hoffmam, according to the figure 1.

$$ln\left(\frac{Abs}{Abs_0}\right) = \mu_{max}.t$$

$$c = \left[\frac{\frac{(AS)_m}{(AA+AF)}}{\mu_m}\right] - \left[\frac{\frac{(AS)_{wt}}{(AA+AF)}}{\mu_{wt}}\right]$$

Figure 1 Hoffmam equation to calcule the maximum growth and the c value

3 RESULTS & DISCUSSION

With screening in microplates, we can obtain results related to the growth of the clones and the maximum growth rate (µmax) and compare them with the original and adapted strain. We can observe these data from Figure 2, 3 and 4.

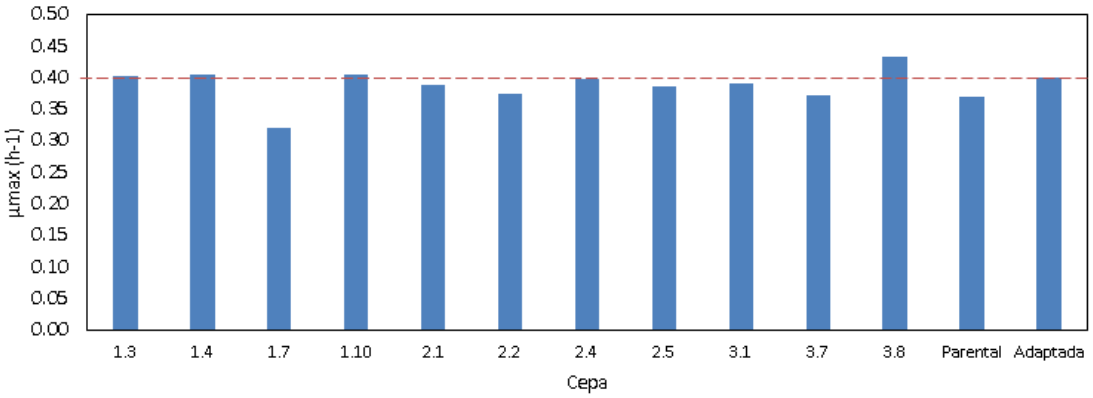

Figure 2 Maximum growth rate (µmax)

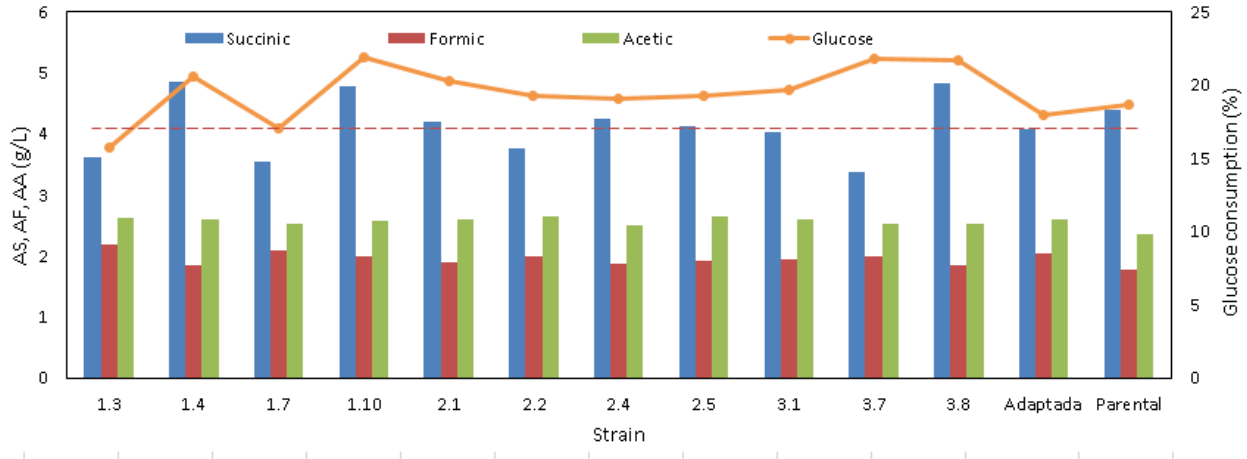

Figure 3 Organic acid production and glucose consumption in microplate fermentation.

It is possible to observe in Figure 2 that the adapted strain can achieve a maximum growth rate of $0.4h^{-1}$, higher than the parental strain. It is also noted that clones 3.8, 1.4, and 1.10 exhibit a higher maximum growth rate than the adapted strain. In order to effectively compare improvements and select the best mutant for future development, the coefficient of comparison of the obtained mutants, or "c" value, was calculated. After the calculations, if the value of C is positive in relation to the adapted strain, it means that it is better in terms of succinic acid production compared to the by-products.

It is possible to note, through Figure 4, that only three clones showed a negative result (1.3, 2.2, and 3.7), and eight clones obtained a positive result. This indicates a positive outcome for the present project.

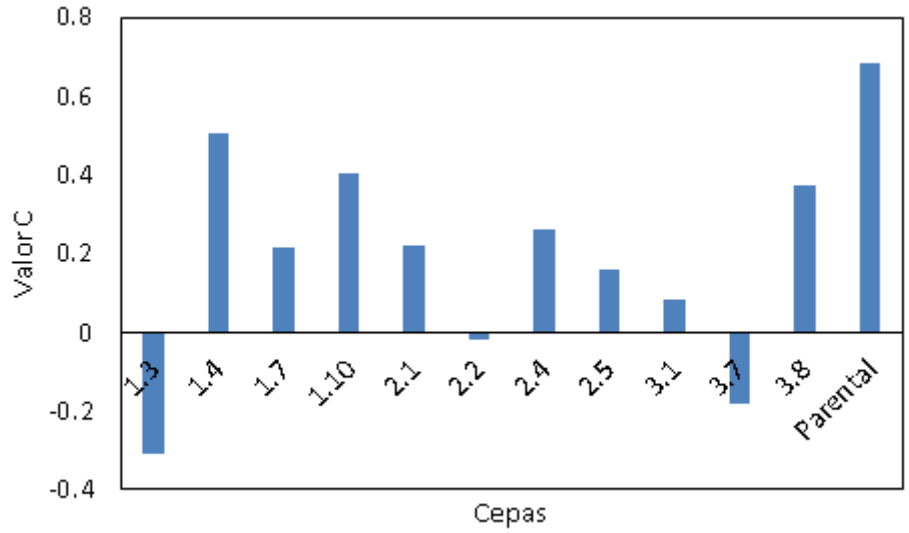

Figure 4 "c" value calculated for comparison of the performance of the clones in succinic fermentation.

4 CONCLUSION

The present project generated positive results regarding the established objectives. Through the calculation of the "C" value, we can conclude that it was possible to develop mutants that exhibit better performance in microplate assays compared to the adapted strain. It is therefore concluded that random chemical mutagenesis with EMS was effective in improving the bacterium *A. succinogenes* for succinic acid production. However, further fermentations in bioreactors with the developed clones are still necessary.

REFERENCES

1 LEE, P.C.; LEE, Q.G.; LEE, A.Y.; CHANG, H.N.; CHANG, Y.K. Fermentative Production of Succinic Acid from Glucose and Corn Steep Liquor by Anaerobiospirillum succiniciproducens. Biotechnol Bioprocess Eng, 5:379-381, 2000.

2 Song, H.; Huh, Y.S.; Lee, S.Y.; Hong, W.H.; Hong, Y.K. Recovery of succinic acid produced by fermentation of a metabolically engineered Mannheimia succiniciproducens strain. J Biotechnol, 132:445–52, 2007.

3 WILLKE, T.H. & VORLOP,K.D, 2004. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. App. Microbiol Biotechnol, v. 66, p. 131-142.

4 BORGES, E. R. Development of a biotechnological process for succinic acid production by *Actinobacillus succinogenes*. p. 226, 2011.

5 FREIRE, F. Production of succinic acid by *Actinobacillus succinogenes* from xylose. Fernanda Freire do Nascimento Student of Biotechnology Production of succinic acid by *Actinobacillus succinogenes* from xylose. Rio de Janeiro. 2010.

6 COSTA, Patrícia dos Santos. Increase in glycohydrolase activities produced by Thichoderma Harzianum by optimizing culture medium and classic genetic improvement aiming at 2G ethanol viability. Campinas, SP: [s.n.], 2017.

7 Hoffmam, Z. B., et al. Evolutionary engineering and chemical mutagenesis of Propionibacterium acidipropionici for improved propionic acid production from sugarcane-derived saccharides, Process Biochemistry, Volume 130, 2023, Pages 584-594, ISSN 1359-5113, https://doi.org/10.1016/j.procbio.2023.05.022.

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