Kinetic Study of Fig Syrup Fermentation by Genetically Modified Saccharomyces cerevisiae Yeast Sstrains. A Physicochemical Approach to the Yeast Strain Life Cycle

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Article

Kinetic Study of Fig Syrup Fermentation by Genetically Modified *Saccharomyces cerevisiae* Yeast Strains. A Physicochemical Approach to the Yeast Strain Life Cycle

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Abstract: Reversed Flow Gas Chromatography (R.F.G.C.) was employed to assess the impact of genetic modification on Saccharomyces cerevisiae yeast strains during the process of alcoholic fermentation, utilizing fig syrup. Multiple fermentations were carried out at various temperatures to evaluate the influence of genetic modifications on yeast strain efficiency. The study involved a wild-type yeast strain, W303, as a control, and two genetically modified strains, W_M4_533 and W_M4_558, sharing the same genetic background as the wild type. Notably, the genetic modifications in the Msn4p transcription factor involved the substitution of serine residues with alanine at positions 533 and 558, resulting in the development of psychrophilic or ethanol-resistant strains. Utilizing the R.F.G.C. method enabled the differentiation of the duration of alcoholic fermentation phases, providing insights correlated to the yeast cell life cycle. The values of rate constants (k) for each phase, conducted with both wild-type and genetically modified cells using RFGC, aligned with existing literature. Additionally, the calculation of activation energies for distinct phases revealed lower values for genetically modified strains compared to wild-type strains. This decrease in activation energies suggests enhanced efficiency in the alcoholic fermentation process for the genetically modified strains.

Keywords: alcoholic fermentation; kinetic study; yeast life cycle; genetic modification; *Saccharomyces cerevisiae;* reversed flow gas chromatography; kinetic study; rate constants; activation energy; fig syrup

1. Introduction

The alcoholic fermentation process stands as a highly scrutinized and industrially pertinent phenomenon, notable for its substantial economic implications within both the food industry and biofuel production domains [1–3]. Numerous variables influence this intricate procedure, offering avenues for enhanced efficiency at both individual and synergistic levels. Considerations encompassing organic matter composition, yeast selection, yeast application methodologies, and tank reactor conditions have been exhaustively investigated [4–7]. Another pivotal facet contributing to the overall sustainability of this process pertains to the economic and environmental ramifications associated with residual materials [8].

Of paramount significance among the determinants of alcohol production efficiency is the yeast employed. Yeasts, functioning as biocatalysts, have undergone comprehensive examination to augment their alcohol-yielding capacity, survivability, and reusability in successive processes [9,10]. In this context, *Saccharomyces cerevisiae*, the preeminent yeast species in alcoholic fermentation, serves as the primary candidate from which various strains are derived. The optimization of this approach has been significantly advanced through biotechnological interventions [11]. A focal point of biotechnological yeast modification lies in bolstering its resilience under challenging conditions and diverse environments [12,13]. To attain this objective, the imperative regulation of genes governing the yeast cell's response to stress conditions becomes requisite. Transcription factors Msn2p and Msn4p emerge as pivotal and indispensable regulators orchestrating the cellular stress response. The intricate mechanism involves these transcription factors binding to stress-response elements (STREs),

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thereby instigating the activation of a repertoire exceeding 200 genes. Notably, investigations into the quantitative transcription dynamics of a yeast strain manifesting ethanol tolerance underscore the substantive role of Msn4p as a regulator crucial for ethanol tolerance [14–17].

Apart from the aforementioned critical parts in the study of alcoholic fermentation, a key aspect involves understanding the life cycle of yeast strains, along with the intricate task of determining kinetic parameters for this process. The determination of these parameters poses a particular challenge, particularly during the initial stages of the alcoholic fermentation procedure.

In this investigation, two yeast strains, denoted W_M4_533 and W_M4_558, were assessed as biocatalysts. These strains underwent genetic modification through the substitution of serine residues in the Msn4p transcription factor with alanine at positions 533 and 558, respectively. The purpose of these alterations was to eliminate the kinase's phosphorylation capacity at these specific sites, thereby facilitating the nuclear entry of Msn4p [18,19]. The yeast strains, W_M4_533 and W_M4_558, alongside the wild-type (W.T.) W303 strain of *Saccharomyces cerevisiae*, will serve as biocatalysts in the alcoholic fermentation of syrup extracted from dried figs [20].

Figs (Ficus carica L.) are fruits of elevated glycemic indexes. Their predominant carbohydrate composition consists of glucose and fructose, with a slightly higher prevalence of glucose (approximately 50% vs. 47%). Figs stand out as a rich source of essential nutrients, including calcium, fiber, copper, manganese, magnesium, potassium, and vitamin K. Additionally, they are abundant in flavonoids and polyphenols, such as gallic acid, chlorogenic acid, syringe acid, and rutin. Amino acids are also present in substantial amounts, rendering figs an adequate nitrogen source for yeasts involved in alcohol and ester production, particularly in alcoholic beverage fermentation.

The selection of figs as a carbon source for biocatalysts is justified by their cost-effectiveness, widespread utilization in the food industry, and the enriched sugar composition [21,22]. Significantly, the utilization of dried figs, readily available in commercial markets, offers the advantage of prolonged preservation of their intrinsic properties [23]. This formal restatement emphasizes the nutritional richness and suitability of figs as a carbon source for biocatalytic processes, particularly in the context of their applications in fermentation and food industries.

The physicochemical assessment of the aforementioned yeast strains for the fermentation of must derived from dried figs will be conducted through a series of experiments, encompassing kinetic analyses under various conditions [24]. To this end, the Reversed Flow Gas Chromatography Technique (R.F.G.C.), a sub-technique of inverse gas chromatography (IGC) [25], will be employed to derive kinetic parameters related to activation energies and rate constants governing the alcoholic fermentation process. This versatile technique, which has found application in diverse studies, including the investigation of diffusion coefficients, mass transfer coefficients, solubility and interaction parameters, adsorption equilibrium constants, rate constants, activation energies, and transformations in surface-catalyzed reactions [26–38], is particularly suited for the kinetic scrutiny of alcoholic fermentation [37,38].

2. Materials and Methods

2.1. Materials

2.1.1. Yeast strains

In this study, *Saccharomyces cerevisiae* yeast was employed in three distinct strains, namely the wild type W303 (wt) and its genetically modified derivatives W_M4_533 and W_M4_558. The modification and isolation procedures for these strains were executed following established protocols [18,19].

All yeast strains were maintained in a liquid medium characterized by the following w/v composition: 2% glucose, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.5% $MgSO_4$ 7H2O, and 0.4% yeast extract, diluted in triple distilled water with a pH value of 5. The chemicals used were procured from Merck, Germany, and the triple distilled water was sourced from laboratory production. To prevent microbiological contamination, the nutrient solution underwent sterilization for 15 minutes at a

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pressure of 1.5 atm and 130 °C. Subsequently, 10 µL of purified yeast was inoculated into a Falcon plastic tube containing 50 ml of the liquid medium. The Falcon tube was then placed in a mechanical incubator within a controlled chamber set at 30°C for 12 hours, with continuous stirring at 200 rpm [39].

2.1.2. Dried Figs Syrup

Dried figs were procured from the local market and subjected to syrup extraction via the hot method. The dried figs underwent segmentation into smaller pieces, enclosed in permeable cloth packages of 1 kg each. These packages were immersed in 1 L of hot water and left undisturbed for 24 hours to facilitate the extraction of hydrocarbons, specifically sucrose and fructose. The liquid's density in Baume (°Be) was subsequently gauged. If the measured density fell below the targeted value of 12.5 °Be, compression of the packages ensued, and the beaker was reheated to augment extraction efficiency. Upon completion of the extraction process, the liquid underwent filtration and sterilization at 134 °C for 30 minutes in an autoclave.

2.2. Apparatus and Procedure

2.2.1. Alcoholic Fermentation in a Laboratory Scale

The incubation protocol outlined in Section 2.1.1 was implemented for each yeast strain (W303, W_M4_588, and W_M4_533). For inoculation, 10^8 cells were utilized in volumetric flasks, each containing 200 ml of sterilized syrup extracted from figs. The fermenting flasks were then housed in a thermostat set to temperatures of 12, 18, and 25 °C. To investigate the kinetics of the alcoholic fermentation process, a 1 ml aliquot of the fermented syrup was extracted and introduced into a glass vessel, connected at the terminus of the diffusion column within the R.F.G.C. chromatograph.

2.2.2. R.F.G.C. Apparatus

The experimental configuration of the Reversed Flow Gas Chromatography (R.F.G.C.) technique is depicted in Figure 1. Two conventional gas chromatographs, namely Pye Unicam Series 104 and Shimadzu 8A equipped with a flame ionization detector, were employed. A stainless steel sample column, possessing an internal diameter of 4 mm and a total length of 2 m, devoid of any packing material, was connected to the diffusion column at the midpoint within the oven of the initial chromatograph. The sample column is bifurcated into two identical sections, denoted as l and l' (each 100 cm + 100 cm).

The diffusion column is comprised of two sections, labeled as z and y (refer to Figure 1). Section z extends for 45 cm and shares the same diameter as the sampling column, whereas section y is a glass vessel measuring 4 cm in length, also with the same diameter as the other columns.

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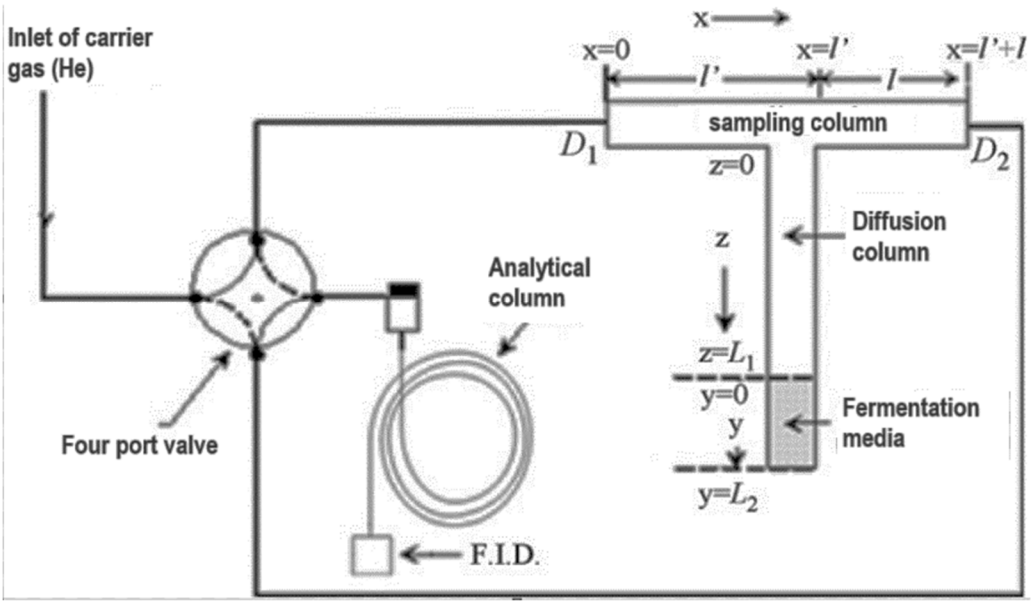

Figure 1. Experimental Configuration of Reversed Flow Gas Chromatography (R.F.G.C.).

To enable versatile control over carrier gas flow through the sampling and diffusion columns, constituting the "sampling cell," the carrier gas input and detector were affixed to these columns. A four-port valve, connecting the ends $x = 0$ and $x = l + l'$ of the sample column to the carrier gas supply and detector, facilitated the ability to alter the direction of carrier gas flow at will. The reversal was sustained for a duration of 6 seconds before returning to its initial direction.

The operational temperature of the sampling cell was maintained at 80 °C. Additionally, to discern the ethanol eluted fraction from potential volatile by-products in the fermented sample, an analytical column was introduced before the detector. This column (2 m × 1/4 in I.D.× 2 mm thick glass) was packed with 5% Carbowax-20 M and 80/120 mesh Carbopack BAW, and maintained at 110 °C within the oven of the second gas chromatograph, equipped with the Flame Ionization Detector (F.I.D.) as illustrated in Figure 1.

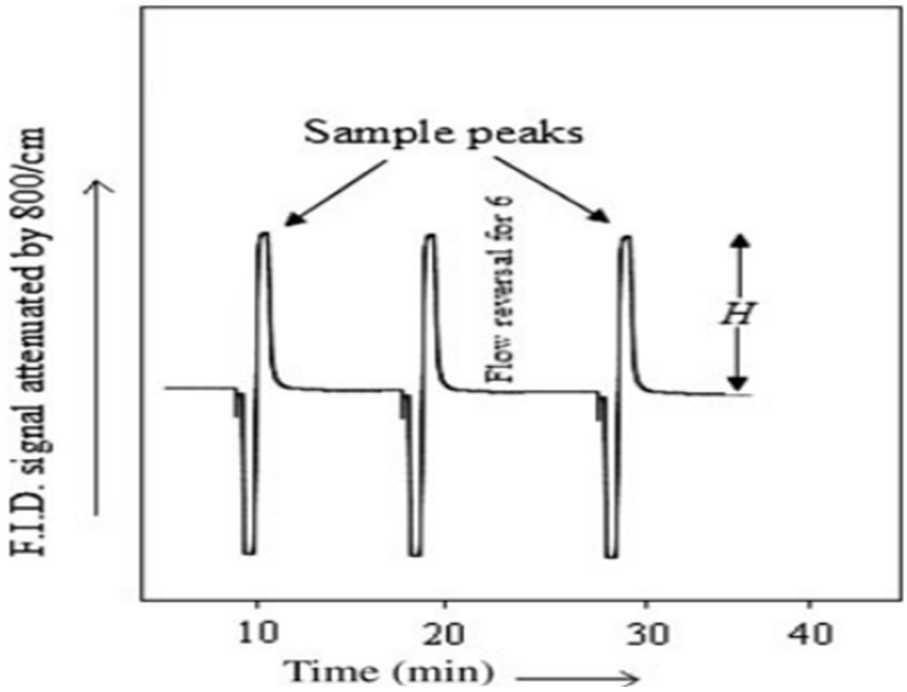

Figure 2. Reversed-flow gas chromatograph showing sample peaks of ethanol at 291.15 K.

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A typical segment of a chromatogram, as depicted in Figure 2, illustrates sample peaks corresponding to ethanol obtained from the alcoholic fermentation of dried figs syrup (12.5 °Be) at 18 °C, featuring the involvement of a wild type of *S. cerevisiae*.

For detector operation, high-purity hydrogen (99.999%) was sourced from Aeroscopio S.A. (Athens, Greece), and atmospheric air was supplied through a compressor apparatus. The detector's operational temperature was set at 150 °C.

Helium served as the carrier gas in all experiments, maintaining a constant flow rate of 0.5 cm^3 s^{-1} with a 1.6 atm pressure drop across the sampling cell. The helium used, obtained from Aeroscopio S.A. (Athens, Greece), was of 99.999% purity.

3. THEORY

The observed sample peaks' height, denoted as H, pertinent to the concentration of the studied gaseous substance, c, near the intersection of the sample column and the diffusion column ($x = l'$), at the time of each flow perturbation (t_0), is approximated by:

$$H \cong 2\, c\,[l', t_0] \qquad (1)$$

where $c\,(l', t_0)$ is the solute concentration at $x = l'$ and at time t_0.

The gaseous substance that comes out from a liquid phase, which is positioned near the bottom of the diffusion column transports until the point $x = l'$, of the sampling column. The only procedure of this mass transport is the diffusion phenomenon and is described from the Fick's second Law:

$$\frac{\partial c}{\partial t_o} = D\,\frac{\partial^2 c}{\partial z^2} \qquad (2)$$

Utilizing Fick's second law and solving this equation under the defined initial condition $c(z, 0) = 0$, the boundary conditions in the diffusion column at $z = 0$, $c(0, t_0) = c(l, t_0)$ and $-D(\partial c/\partial z)_{z=0} = uc(l, t_0)$ and the boundary conditions at $z = L$, $D(\partial c/\partial z)_{z=L} = k_c(c_0 - c_{z_L})$, the expression of the substance concentration at the junction point of the diffusion and the sample column at various times is derived [41].

$$c\,[l', t_0] = \frac{k_c D c_o}{v\,(k_c L + D)}\left\{1 - \exp\left[-\frac{2(k_c L + D)t_0}{L^2}\right]\right\} \qquad (3)$$

where c_0 is the concentration of the substance at the gas – liquid interphase in equilibrium with the bulk liquid phase, c_{z_L} is the concentration of the substance at the boundary layer of gas – liquid interphase in equilibrium with c_0, k_c is the mass transfer coefficient for the evaporation process, D is the diffusion coefficient of the substance into the carrier gas, v is the linear velocity of the carrier gas and L is the length of the diffusion column in the R.F.G.C. system.

Combination of Eqs 1 and 3 yields:

$$H = 2\frac{k_c D c_o}{v\,(k_c L + D)}\left\{1 - \exp\left[-\frac{2(k_c L + D)t_0}{L^2}\right]\right\} \qquad (4)$$

Equation 4 predicts the elution curve's form. Moreover, an infinity value of H_{max} can be deduced, for extended time periods [30]:

$$H_{max} = \frac{2k_c D c_o}{v\,(k_c L + D)} \qquad (5)$$

Here, H_{max}, signifies the sample peak height at the plateau region of the elution curve. Notably, this expression indicates that peak height is inversely proportional to the concentration of the substance at the gas–liquid interphase, a quantity analogous to the substance's bulk concentration.

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Measured values of H_{max}, can be utilized in a suitable mathematical expression for the kinetic phenomenon under investigation. For alcoholic fermentation, a first-order reaction rate expression aligns well with experimental results. The modified model, incorporating experimental peak height values, is expressed as follows [42]:

$$\ln(H_{max\infty} - H_{\max}) = \ln\ (H_{\infty}) - k\ t_0 \qquad (6)$$

Here, $H_{max\infty}$, denotes the sample peak value when the fermentation process concludes at extended times, and k represents the first-order rate constant for the alcohol-producing reaction.

4. Results and Discussion

4.1. Ethanol Production Ability

To assess the efficiency of alcoholic fermentation for each strain (W303 wild type, W_M4_558, W_M4_533), samples collected at various time points during the fermentation process were subjected to analysis using reversed-flow gas chromatography (R.F.G.C.). The peak height, denoted as H, was extracted from the recorded chromatograms, as it has been established that H linearly corresponds to the ethanol concentration c [31]. Specifically, H represents the distance from the baseline to the maximum height at the conclusion of the R.F.G.C. experiment. The results are carried out after three repetition at each temperature and each yeast strain.

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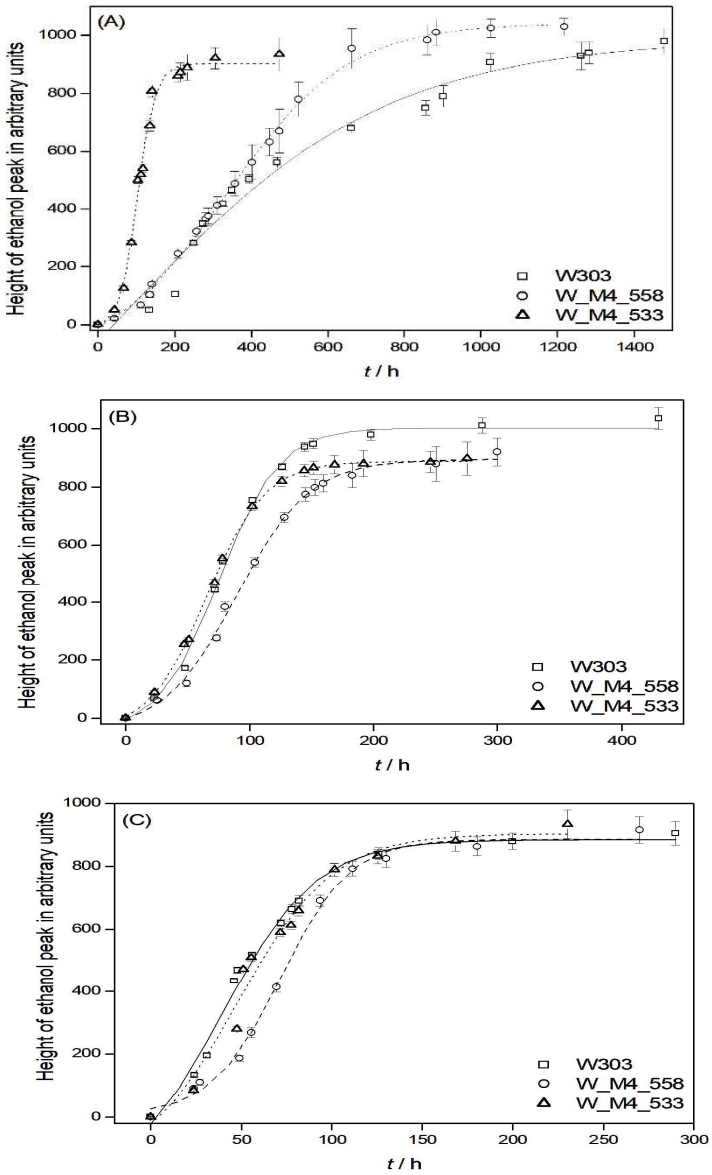

Figure 3. Graphical representation of ethanol production, illustrating the relationship between the peak height (*H*) and time, accompanied by sigmoidal fitting curves for each strain. In each graph the error bars after three replicates are also given. Alcoholic fermentation was conducted at A) 12°C, B) 18°C, and C) 25°C.

The graphical representation of *H* versus time at a fermentation temperature of 12°C revealed that the substitution of the serine residue with alanine at position 533 of the Msn4p transcription factor, while not influencing ethanol production, results in a threefold reduction in the time required for completion compared to the wild-type strain. Moreover, the substitution of the serine residue with alanine at position 558 demonstrates a two-and-a-half-fold decrease in the time required for completion compared to the wild-type strain (Figure 3A). Those can be attributed to the fact that the genetic modifications of the wild type leads to more psychrophilic and alcoholic-resistant yeast strains.

At temperatures of 18°C and 25°C, both wild-type and genetically modified yeast strains exhibit a shorter duration for alcoholic fermentation compared to the time required at 12°C, as anticipated [43]. Upon comparing genetically modified yeasts with the wild-type strain, it is discerned that, despite the genetically modified strains displaying a shorter fermentation duration than the wild type, these differences do not attain statistical significance (Figure 3B and 3C).

The above findings do not entirely align with previously published results [44] regarding the use of the same genetically modified yeasts in fermenting grape must. While the outcomes for strain

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W_M4_533 exhibit similarities, there are notable discrepancies in comparison to the other modified strain W_M4_558.

4.2. Kinetic Analysis

Alcoholic fermentation is characterized by four distinct stages from the beginning to the end of the fermentations, and they correspond to the phases of the alcoholic fermentation processes. Sometimes three distinct stages observed as the absorption of one phase by its neighboring phase. The kinetic characteristics of each phase were calculated by plotting ln (H_∞ - H) against t. Here, H_∞ represents the peak height of the sample collected at the end of fermentation, and h corresponds to the peak height of each sample collected at time point t. Rate constants (k) were determined for each stage of the fermentation process. Representative plots for each strain at 18°C are depicted in Figure 4, illustrating three distinct phases of alcoholic fermentation corresponding to the lag phase, log phase, and stationary phase. These plots confirm that alcoholic fermentation can be modeled as a pseoudo-first-order reaction.

As a result, the above mentioned slopes correspond to the lag, log and stationary phases. The slopes were verified by r^2 values between 0.94 and 1.00. From the slopes of the above mentioned straight lines and remembering Eq. (6) the values of rate constants of ethanol production, k, can be easily calculated for the three phases observed during the alcoholic fermentations conducted with wild type yeast strains as well as genitically modified strains at various temperatures. Since the rate constants for ethanol production coincide with the phases of the alcoholic fermentation process, the duration of each of the aforementioned phases can easily be estimated. The results are presented in Table 1 and they are mean values calculated after three replications for each system. Their corresponding standard deviations are given as well.

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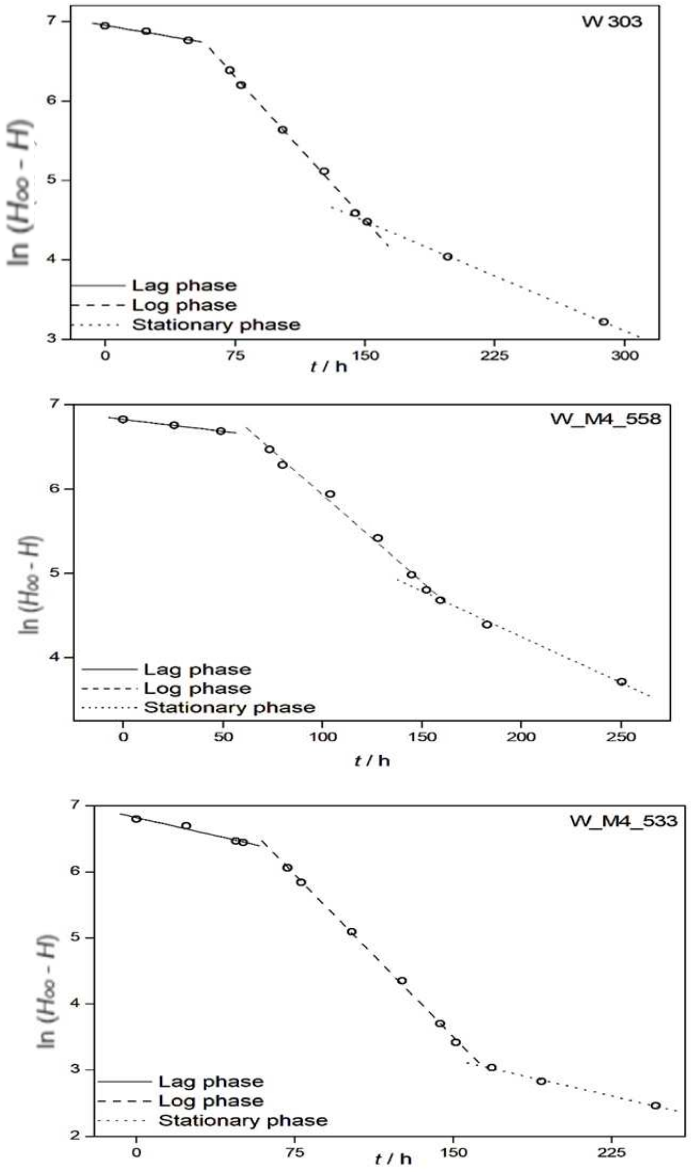

Figure 4. Kinetic Analysis of Alcoholic Fermentation. Representative plots depicting ln(H_∞ - H) against time for fermentation at 18°C elucidate three discernible phases of fermentation: lag, log, and stationary. The slope of each line corresponds to the reaction rate constant (k).

In Table 1 the reaction rate constants (k) for each phase (lag, log, and stationary) at various temperatures, along with their respective standard deviation are illustrated.

Table 1. Mean values of rate constants for ethanol production, k, with their corresponding standard deviations (σ) for the three fermentation phases for each one of different yeast strains at different fermented temperatures.

Yeast strain	θ/ °C	$(k \pm \sigma)$ (10^3 h^{-1})		
		Lag phase	Log phase	Stationary phase
W303	12	0.54±0.13	1.83±0.07	1.95±0.64
	18	2.19±0.49	7.02±0.48	5.25±0.09
	25	7.49±0.99	20.92±0.99	12.93±0.45
W_M4_558	12	0.91±0.18	3.39±0.24	7.27±2.12
	18	2.59±0.08	10.34±0.77	9.52±0.48
	25	6.83±0.14	27.43±1.43	11.95±1.18

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W_M4_533	12	1.87±0.53	7.72±2.27	5.36±0.56
	18	3.70±0.91	16.27±0.85	9.17±0.67
	25	7.48±1.98	35.47±1.54	14.67±0.11

From the results presented in Table 1, one can derive the following insights:

- Throughout the fermentation process at 12°C, the lag phase revealed an approximately twofold increase in the reaction rate constant for strain W_M4_558 and a more than threefold increment for W_M4_533, both in comparison to the wild-type strain. These disparities were found to be statistically significant ($p < 0.05$, 1-way ANOVA). In the log phase, both genetically modified strains exhibited heightened reaction rate constants. Specifically, the Ser558Ala modification resulted in a statistically significant 1.5-fold increase in k ($p < 0.05$, 1-way ANOVA), while the Ser533Ala modification proved notably more effective, yielding an almost fourfold increase in the reaction rate constant ($p = 0.05$, 1-way ANOVA). Moving to the stationary phase, both genetically modified strains demonstrated increased reaction rate constants compared to the wild type. W_M4_558 exhibited a three-and-a-half-fold greater increase ($p < 0.05$, 1-way ANOVA), and W_M4_533 showed a two-and-a-half-fold increment ($p < 0.05$, 1-way ANOVA). In summary, the Ser533Ala substitution conferred the strain with efficient fermentation capabilities at low temperatures. Furthermore, while the Ser558Ala substitution did not manifest increased fermentative ability during the log phase, it exhibited enhanced capability during the stationary phase, particularly in the presence of elevated ethanol levels in the medium, suggesting a potential ethanol-resistant phenotype at low temperatures.
- In the context of fermentation at 18°C, there is an increment in the *k* value during the Lag phase for both genetically modified yeasts when compared to wild-type yeasts; however, these differences do not attain statistical significance at $p < 0.05$, 1-way ANOVA. Conversely, during the subsequent log and stationary phases, the observed enhancements in the *k* constant values are statistically significant ($p < 0.05$, 1-way ANOVA).
- This observation may be ascribed to the assumption that genetic modification does not manifest a discernible impact on the rate of yeast adaptation to the fermentation environment at 18°C—a condition intrinsically stressful for the yeasts. After that, the fermentation efficiency of the genetically modified yeasts appears to be significantly augmented owing to the specific genetic modifications implemented, as contrasted with the wild-type strains.
- Similar observations to those at 18 ^{0}C are drawn for fermentations at 25 ^{0}C regarding the behavior of the yeasts across the three phases of alcoholic fermentation. This further strengthens the conclusion that at elevated temperatures, genetic modifications impact the fermentation capacity by reducing fermentation time, while not affecting the duration of the yeasts' adaptation to the fermentation medium.
- These observations align with the observed duration of alcoholic fermentation. As noted, the most significant impact of genetic modification was evident during fermentation at 12 ^{0}C, while the effects at 18 ^{0}C and 25 ^{0}C were not statistically significant.
- The results in Table 1 are in the same order of magnitude with previous works. In a study carried out by Ozilgen et al. [45] the rate constant for the Lag phase have been calculated between 0.024 h^{-1} (fast fermentation) and 0.006 h^{-1} (slow fermentation), while the rate constant for the stationary phase have been calculate between 0.039 h^{-1} (fast fermentation) and 0.042 h^{-1} (slow fermentation), also Giovanelli et al. [46] reported, a specific growth rate of *S. cerevisiae* equal to 0.13 h^{-1} under aerobic conditions and 0.07 h^{-1} under anaerobic conditions.

Utilizing the Arrhenius equation, the activation energies (E_α) for various stages of alcoholic fermentation were determined:

$$\ln(k) = \ln(A) - \frac{E_a}{R}\frac{1}{T} \qquad (7)$$

Here, *k* represents the reaction rate constants of the alcoholic fermentation phases, *A* is the pre-exponential factor, E_α is the activation energy, *R* is the gas constant, and *T* is the temperature. Activation energies for each stage of the fermentation process were calculated from the slopes of the

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graphical representations using the determined values of rate constants for each stage of alcoholic fermentation. The results are illustrated in Table 2.

Table 2. Activation energies E_α with their corresponding standard deviation (σ) for each phase of alcoholic fermentation for each yeast strain, computed from Arrhenius equation plots.

	E_α (KJ/mol)		
Yeast strain	Lag phase	Log Phase	Stationary phase
W303	142.7 ± 9.9	132.1 ± 12.1	102.7 ± 6.0
W_M4_558	109.4 ± 5.8	113.4 ± 7.9	26.9 ± 2.2
W_M4_533	75.3 ± 1.8	82.9 ± 1.5	54.6 ± 3.9

It appears that the results presented in Table 2 align with previously drawn conclusions. Specifically, the activation energy during the lag phase for the wild-type yeast strain is nearly double that of the activation energies observed for the W_M4_533 genetically modified strain. Additionally, the activation energy for the wild-type strain during the lag phase is approximately half as large as the activation energy for the W_M4_558 modified strain.

The lag phase is crucial as it characterizes the adaptation of yeast strains to the fermented media. Notably, the genetically modified strains (W_M4_533 and W_M4_558) exhibit smaller activation energies during the lag phase compared to the wild-type strain. This suggests that the genetic modifications have an impact on the yeast strains' ability to adapt to the fermented media during this phase.

Furthermore, in the two other phases considered, the genetically modified strains also show smaller activation energies compared to the wild-type strain. This implies that the genetic modifications have a consistent effect of reducing activation energies in these phases, indicating potential improvements or alterations in the metabolic processes of the modified strains.

5. Conclusions – Featured Applications

The fermentation process was meticulously monitored utilizing the RF-GC technique, enabling the assessment of different yeast strains' proficiency in fermenting fig syrup. Beyond physicochemical quantities related to alcohol production, this technique facilitated the estimation of various phases within the yeast life cycle.

Althought conventional Gas Chromatography (GC) with Flame Ionization Detector (FID) or Mass Spectrometry (MS) detection has been widely employed for quantifying ethanol concentration and by-products in fermentation processes, Reversed Flow Gas Chromatography (R.F.G.C.) emerges as a viable alternative. R.F.G.C. proves effective not only in determining ethanol concentration but also in assessing physicochemical parameters associated with the kinetics of each phase in alcoholic fermentation. This suggests that the application of R.F.G.C. allows for a more in-depth analysis, possibly capturing nuances and details that go beyond traditional concentration measurements. The information gathered through R.F.G.C. appears to be particularly relevant for assessing the efficiency of fermentation, especially in the context of genetically modified yeast strains.

Regarding to the substitution of a serine residue with alanine at position 533 (strain W_M4_533) suggested that this particular serine residue potentially inhibits the fermentation ability of *S. cerevisiae* at various temperatures. Interestingly, this substitution conferred an enhanced fermentation ability at lower temperatures, significantly reducing the total fermentation time without compromising alcohol synthesis efficiency. The Ser558Ala substitution (strain W_M4_558) also contributed to a reduced fermentation time, albeit to a lesser extent, gaining efficiency in the latter half of the fermentation process.

In order to confirm the observed behaviors and draw robust conclusions about the effects of genetic modifications on fermentation efficiency, it would be advisable to conduct fermentations in various fermentation media. In-depth kinetic analysis of each fermentation phase revealed that the

W_M4_533 strain exhibited superior performance during the lag and log phases at lower temperatures, indicative of a psychrophilic phenotype. Conversely, the W_M4_558 strain demonstrated enhanced fermentation efficiency during the stationary phase at lower temperatures, hinting at a potential ethanol-resistant phenotype.

Absolutely, assessing the impact of genetically modified yeasts on the quality characteristics of the produced beverages requires a comprehensive analysis that goes beyond concentration measurements and fermentation efficiency. To draw meaningful conclusions, parameters such as aroma and taste profile, by-products production, consistency and reproducibility, microbial stability etc should be determined, in order the final product meets the regulatory standards for alcoholic beverages.

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