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Fermentation of Sugar by Thermotolerant *Hansenula polymorpha* Yeast for Ethanol Production

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Abstract: *Hansenula polymorpha* is a non-conventional and thermo-tolerant yeast that is well-known for its use in the industrial production of recombinant proteins. However, research to evaluate this yeast's potential for the high-temperature fermentation of sugar to produce alcohols for biofuel applications is limited. The present work investigated a wild-type *H. polymorpha* strain (DSM 70277) for the production of ethanol at a temperature of 40 °C under limited oxygen presence by using a batch fermentation reactor. Fermentation experiments were performed using three types of sugar (glucose, fructose, xylose) as substrates with two initial inoculum concentrations (1.1 $g{\cdot}L^{-1}$ and 5.0 $g{\cdot}L^{-1}$). The maximum specific growth rates of *H. polymorpha* yeast were 0.121–0.159 h^{-1} for fructose, 0.140–0.175 h^{-1} for glucose, and 0.003–0.009 h^{-1} for xylose. The biomass volumetric productivity was 0.270–0.473 $g{\cdot}L^{-1}h^{-1}$ (fructose), 0.185–0.483 $g{\cdot}L^{-1}h^{-1}$ (glucose), and 0.001–0.069 $g{\cdot}L^{-1}h^{-1}$ (xylose). The overall yield of ethanol from glucose (0.470 $g{\cdot}g^{-1}$) was higher than that from fructose (0.434 $g{\cdot}g^{-1}$) and xylose (0.071 $g{\cdot}g^{-1}$). The *H. polymorpha* yeast exhibited different behavior and efficacy regarding the use of glucose, fructose, and xylose as substrates for producing ethanol. The present knowledge could be applied to improve the fermentation process for valorization of waste biomass to produce bioethanol.

Keywords: *Hansenula polymorpha*; glucose; fructose; xylose; fermentation; bioethanol

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1. Introduction

Bioethanol is the term used for ethanol produced from biomass for biofuel applications. Its combustion efficiency is 15% higher than that of gasoline due to the presence of 34.7% oxygen [1,2]. Consequently, the utilization of bioethanol as a substitute for or additive to gasoline has been intensifying to minimize the use of fossil fuels, protect the environment, and promote renewable energy applications [3,4]. Ethanol–gasoline blends are widely used as transportation fuels in Brazil, the United States, and Europe [5–9]. Bioethanol production from inedible lignocellulosic biomass by the integrated use of acid/enzymatic hydrolysis and fermentation methods is a promising technology [10,11]. The lignocellulosic biomass is generally pre-treated through acid or enzymatic hydrolysis, auto-hydrothermal, solvothermal, and steam explosion methods to disintegrate recalcitrant polymeric lignocellulosic fractions. This produces a liquid hydrolysate rich in monomers or simple sugar that is fermented to produce bioethanol [12–17]. These pre-treatment methods are currently used by several companies for the industrial production of cellulosic bioethanol [1]. Based on the treatment conditions, the yield and type of simple sugar vary, which mainly include glucose and xylose in higher quantities along with other sugars such as fructose, sucrose, mannose, galactose, arabinose, cellobiose, maltose, etc. [18–20].

Fermentation of sugar using yeasts is an established and widely used method for the production of alcohols [21]. During fermentation, the yeast cells metabolize sugar and transform it into ethanol, carbon dioxide, and heat through successive enzymatic

actions [22,23]. The *Saccharomyces cerevisiae* yeast is conventionally used in the fermentation process due to its high ethanol production, tolerance for a broad pH range, and high ethanol concentration [24,25]. The major challenges in the conventional fermentation process are the rise in temperature (35–45 °C) and ethanol content (above 20%), which inhibit the growth and productivity of yeasts. The metabolic activities of microbes generate heat stress during the fermentation process, which causes defects in and the reduced growth of microbes. Therefore, chiller systems are used to cool and maintain a suitable temperature in the fermentation reactor [26]. The *Saccharomyces cerevisiae*-based conventional fermentation involves additional cooling system requirements due to its limited thermotolerance capacity (30 °C). High-temperature (40–45 °C) fermentation is an emerging process that uses non-conventional thermotolerant yeasts like *Hansenula polymorpha* to produce ethanol from lignocellulose sugar (mainly xylose) [27–29]. This process is considered to be more economical and beneficial as it reduces the risk of microbial contamination and minimizes reactor cooling costs [26,30].

H. polymorpha, or *Pichia angusta*, is part of the *Saccharomycetaceae* fungal family and the *Saccharomycetoideae* subfamily. It is a facultative methylotrophic yeast species and is safe for use because it does not carry pathogens, pyrogens, or viral inclusions. It has the ability to grow using various carbon sources, such as glucose, sucrose, maltose, glycerol, xylitol, and methanol. This makes its cultivation simple and easily upscalable for industries. The *H. polymorpha* cells are robust and can perform fermentation at a temperature of 20–45 °C and a pH range of 2.5–6.0. The industrial application of *H. polymorpha* has been mostly focused on the production of proteins for pharmaceutical (Hirudin) and biocatalyst (phytase and cellulase) applications [31]. However, to realize its application for bioethanol production, more extensive research is required [32]. The research could include investigating high-temperature fermentation processes using different wild-type and mutant strains of *H. polymorpha* for the cost-effective production of ethanol [26,30].

Earlier research studies on *H. polymorpha* strains have demonstrated its ability to ferment lignocellulose sugars at high temperatures for ethanol production. A preliminary study was carried out on different wild-type strains of *H. polymorpha* for the fermentation of glucose and xylose in tube cultivation for ethanol production [32]. The fermentation of sunflower-stalk hydrolysate (produced by acid hydrolysis) by *H. polymorpha* (ATCC 34438) was reported to produce ethanol and xylitol [33,34]. The fermentation of wheat straw hydrolysate (produced by enzymatic hydrolysis) by *H. polymorpha* (CBS 4732) was also reported to produce ethanol [35]. All of these studies have been conducted in varied conditions with different carbon substrates. For instance, the study by Ryabova et al. (2003) reported the fermentation of pure sugars (glucose, xylose, cellobiose, mannose, arabinose, maltose, and galactose) to produce ethanol [36]. The other studies use hydrolysates that contain a mixture of simple sugars and inhibitors. It is reported in the literature that limited oxygen supply improves the fermentation of xylose by *H. polymorpha* for alcohol (ethanol and xylitol) production [37]. Furthermore, existing studies are mostly focused on ethanol production from glucose and xylose. Until now, no study has been reported on the efficacy of *H. polymorpha* to ferment fructose for ethanol production. More research is required to advance our knowledge on the fermentation of *H. polymorpha* for ethanol production from simple sugar.

For the first time, the present study investigated (1) the efficacy of the wild-type *H. polymorpha* strain (DSM 70277) for the fermentation of analytical grade glucose, fructose, and xylose to produce ethanol under limited oxygen conditions; and (2) the influence of inoculum concentration and fermentation time (0 to 100 h) on yeast biomass growth, sugar uptake, and ethanol production. The results will advance research on the development of *H. polymorpha*-based high-temperature fermentation technology under the presence of limited oxygen for ethanol production.

2. Materials and Methods

2.1. Yeasts Procurement and Cultivation

The wild-type *Hansenula (Ogataea) polymorpha* yeast strain (DSM 70277) was procured from the Leibniz Institute, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany. It was transferred to the autoclaved solid agar media composed of 3 $g \cdot L^{-1}$ yeast extract (Oxoid, Thermo Fisher Scientific Inc., Hampshire, UK); 3 $g \cdot L^{-1}$ malt extract (Merck, Darmstadt, Germany); 5 $g \cdot L^{-1}$ peptone (Applichem ITW, Panreac, Darmstadt, Germany); 10 $g \cdot L^{-1}$ xylose (Fagron, Waregem, Belgium); and 20 $g \cdot L^{-1}$ agar–agar (Fagron, Waregem, Belgium), which were all microbiology-grade chemicals.

2.2. Fermentation Experiment

A small amount of yeast was shifted from the solid agar medium to a liquid culture medium. The liquid medium was prepared (Lindegren et al. (1958) [38] by mixing 2.0 $g \cdot L^{-1}$ yeast extract (Oxoid, Thermo Fisher Scientific Inc., Hampshire, UK), 1.8 $g \cdot L^{-1}$ peptone (Applichem ITW, Panreac, Darmstadt, Germany), and analytical-grade chemicals (Panreac, Barcelona, Spain) such as 1.5 $g \cdot L^{-1}$ $(NH_4)_2SO_4$, 1.0 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, and 1.0 $g \cdot L^{-1}$ KH_2PO_4 in double-distilled water. Thereafter, the yeast was cultured in flasks with that liquid medium and subjected to shaking (150 rpm) at 30 °C. After 48 to 72 h, the suspensions were centrifuged at 5000 rpm for 10 min to obtain concentrated yeast biomass concentrations. From this concentrated biomass, different inoculum concentrations were prepared through serial dilutions and used for fermentation experiments.

In order to perform fermentation experiments simultaneously with glucose, fructose, and xylose, a batch bioreactor (LSCI, Languedoc Scientifique Company Limited, Rivesaltes, France) with three borosilicate glass reactors (approx. 1 L capacity) with stirrers and a PID controller (42 series, Chemitec, Florence, Italy) to automatically regulate temperature, pH, and oxygen supply was used. The technical details of this bioreactor are available in the LSCI general catalogue 2019 [39]. In the present work, the fermentation experiments were conducted at 5.5 pH and 40 °C. These conditions were reported in prior studies to be favorable for *H. polymorpha*-based fermentation [33,36]. Solutions of glucose (Panreac, Barcelona, Spain), fructose (Merck, Darmstadt, Germany), and xylose (Fagron, Waregem, Belgium) of different concentrations (12.5–25 $g \cdot L^{-1}$) were prepared using the above-mentioned liquid culture medium. The pH value of these solutions was adjusted to 5.5 using 1N KOH or 1N HCl. All the components used for the fermentation experiments, such as bioreactor vessels and pH electrodes were previously autoclaved (Systec VB-40, Systec GmbH & Co. KG, Deutschland, Germany) at 121 °C for 20 min. Thereafter, sugar solutions were mixed in the liquid culture medium and introduced into the bioreactor vessels. Once the bioreactors' vessels reached 40 °C, a specific inoculum concentration of yeast was fed into them. The water chiller system was also fixed to the bioreactor vessels to prevent evaporative loss of fermentation broth. Aeration was only provided by the stirrer during the fermentation, and yeasts were mainly dependent on the dissolved oxygen present in the nutrient broth for growth [40]. The fermentation experiments were conducted under magnetic stirring conditions (500 rpm) from 0 to 100 h, and a 5 mL sample was periodically removed from the reactor vessels through a plastic syringe for different analyses.

2.3. Analysis of Samples

Dissolved oxygen (DO) concentration in the fermentation media before and after the fermentation experiments were measured using the Optical Dissolved Oxygen Instrument (HI-98198, Hanna Instruments Ltd., Bedfordshire, UK). Approx. 5 mL samples were periodically removed from the fermentation bioreactor and centrifuged and the upper liquid portion was separated; then, distilled water was added to the yeast cells to make up the volume and dilute the sample for the spectrophotometric measurement. The yeast biomass concentration ($g \cdot L^{-1}$) was measured spectrophotometrically using a standard calibration curve prepared using the optical density (OD) value (taken at 620 nm) and its biomass oven-dried (110 °C) weight (g). This method was previously reported by researchers for *H.*

polymorpha [34,40]. Standard protocol by Bray (2003) [41] was used for scanning electron microscopy (SEM) analysis of yeast cells, which is briefly described as follows: A concentrated yeast cells suspension was dehydrated through gradual treatment with 30% (for 12 h) and then 70 to 100% (for 1 h) concentrations of ethanol in a PF17-Automatic Mini Tissue Processor (Lynx II, Aname, Madrid, Spain). Thereafter, the dehydrated sample was dried in a critical point dryer (CPD, CPD 030 Critical Point Dryer, BalTec AG, Pfäffikon, Zurich, Switzerland), followed by the addition of a gold coating (Q150T Plus—Turbomolecular pumped coater, Quorum, East Sussex, UK) on the sample surface for the Scanning Electron Microscopy (Merlin, Carl Zeiss, Oberkochen, Germany) analysis. The liquid portion separated through centrifugation of samples collected during the fermentation was diluted using double-distilled water and analyzed for sugar and ethanol concentrations. The glucose, fructose, and xylose concentrations were analyzed using an ion chromatograph with a Metrosep Carb 2 100/4.0 IC column (Carb 2 150/4.0) and a pulsed amperometric detector (930 Compact IC Flex, Metrohm AG, Herisau, Switzerland). The aforementioned sugars were purchased from Sigma Aldrich brand (Merck, Darmstadt, Germany) and were used for preparing the calibration curve. The eluent used for ion chromatography was a mixed solution of 100 mM NaOH and 10 mM sodium acetate, prepared from the stock solution provided by Metrohm AG company (Herisau, Switzerland). The concentrations of ethanol in samples were analyzed by gas chromatography (Shimadzu 2010 plus, Kyoto, Japan) with AOC-20s autosamplers, an FID detector, and a capillary column (BPX5). Helium was used as a carrier gas with a flow rate of 50 $mL{\cdot}min^{-1}$. Sample analysis was performed utilizing external ethanol (GC standard, Sigma Aldrich, Merck, Darmstadt, Germany) standard for calibration, and the column temperature of 55 °C, 100 Kpa pressure, and a 70 °C oven temperature.

2.4. Mathematical Modelling

The maximum specific growth rate (μ_m) and biomass volumetric productivity (P_b) of the yeast were estimated using Equations (1) and (2) [34,40], respectively. In Equation (1), μ_m = Maximum specific growth rate, h^{-1}; x = cell concentration per volume; and x_0 = cell concentration at the initial time. The μ_m was estimated by considering the slope of the trendline of ln (x/x_0) versus the time curve [40]. On the other hand, in Equation (2), P_b = biomass volumetric productivity ($g{\cdot}L^{-1}h^{-1}$), and c = empirical constant.

$$\ln\left(\frac{x}{x_0}\right) = \mu_m t + a \quad (1)$$

$$x = c + P_b t \quad (2)$$

The uptake of substrates (glucose, fructose, or xylose) was assessed by determining the specific rate of substrate uptake (q_S^D) based on Equation (3) [34]. The q_S^D is the specific rate of substrate uptake by yeast with respect to time.

$$S = S_0 \alpha^{-t^{\beta}} \quad (3)$$

where S = substrate uptake at a specific time; S_0 = substrate uptake at initial time (t = 0); α and β = constants measured using the equation by linearizing and performing least-square adjustments.

Overall biomass yield ($Y_{X/S}^O$), measured as per Equation (4) [33], is a quotient between net biomass produced and net uptake of substrate. In this equation, $Y_{X/S}^O$ = value of slope, calculated from the values of $x - x_0$ (at varied times) versus $S_0 - S$. The $Y_{X/S}^O$ is represented by the slope value obtained from the curve of change in concentrations of yeast at varied times versus change in substrate uptake [33].

$$Y_{X/S}^O = \frac{dx}{d(S_0 - S)} \quad (4)$$

The specific rate and yield of ethanol are estimated using Equations (5) and (6) [33,34], respectively. In Equation (5), X_p = concentration of ethanol; a and b = constants indicating the intercept and slope values, respectively, determined using the least-square linear fitting curve of X_p versus time (t); q_p^D = value calculated by the differential method, based on the equation, whereas in Equation (6), p = concentration of ethanol;Y_P^O (Overall ethanol yield) = value of slope, calculated from the values of $p - p_0$ (at varied times) versus $S_0 - S$. Similarly, instantaneous product yield (Y_p) is measured using the values of $p - p_0$ (at varied times) versus $x - x_0$.

$$X_p = a + bt \quad (5)$$

$$Y_P^O = \frac{d_p - d_{p0}}{d(S_0 - S)} \quad (6)$$

3. Results and Discussion

3.1. SEM Imaging

The SEM images of the *H. polymorpha* yeast strain (DSM 70277) used for fermentation are shown in Figure 1. The images indicated that the yeast cells were of spherical shape and in a healthy condition, free from any contamination from other microbes. Distinctive cells with moderate aggregation were also observed. A similar cellular structure and aggregation were earlier reported for *H. polymorpha* by Manfrão-Netto et al. (2021) [42].

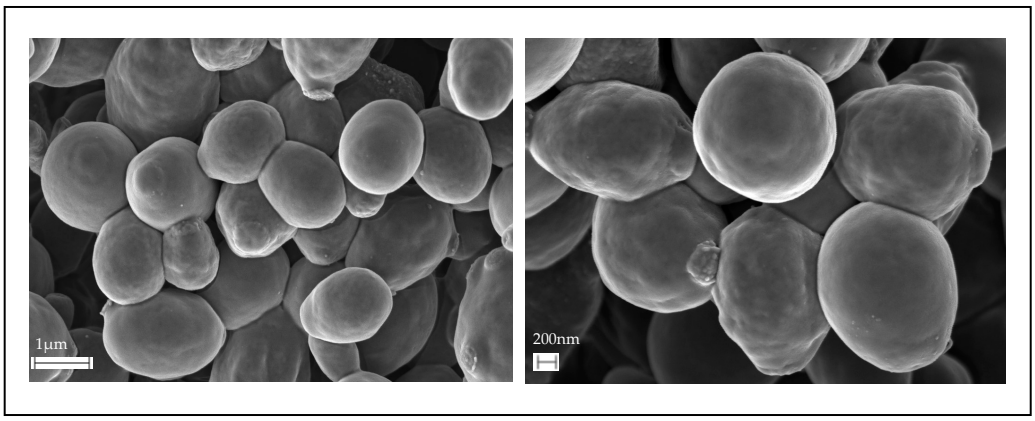

Figure 1. Scanning electron microscopy images of the *H. polymorpha* yeast.

3.2. Dissolved Oxygen Measurements

The DO content in the fermentation broth was measured before (initial) and after (final) the fermentation experiments. The initial medium contained a DO content of 5.53 $mg{\cdot}L^{-1}$, whereas the final media collected after end of fermentation experiments contained DO contents of 1.03 $mg{\cdot}L^{-1}$ (fructose), 1.14 $mg{\cdot}L^{-1}$ (glucose), and 0.69 $mg{\cdot}L^{-1}$ (xylose). The consumption of DO was relatively higher in xylose, whereas there was a very narrow difference between glucose and fructose. The present findings confirm the capability of *H. polymorpha* to grow and perform fermentation under limited aeration conditions [21]. Stöckmann et al. (2003) also reported the growth of *H. polymorpha* and an ethanol production of 4.1 $g{\cdot}L^{-1}$ under a limited oxygen supply using glucose cultures [43].

3.3. Yeast Growth and Productivity

The higher and lower inoculum concentrations used for fermentation experiments were approximately 5.0 and 1.1 $g{\cdot}L^{-1}$, respectively. The inoculum concentrations influenced the growth of the yeast (presented in Figure 2). With a lower inoculum concentration, the log phase started earlier (0.25 h) for fructose and glucose, while it was delayed (0.5 h) for xylose. The log phase was observed after a relatively longer time period with the higher inoculum concentration, which was 0.5 h for fructose and glucose and 16 h for xylose. For glucose, a higher inoculum concentration produced the maximum yeast biomass concentration faster

at 15 h, compared to 32 h with a lower inoculum concentration. In the case of fructose, the maximum yeast biomass concentrations were found after 5 h of fermentation time with an inferior inoculum concentration compared to that after 15 h in the case of the higher inoculum concentration. Similarly, less inoculum showed a maximum biomass concentration sooner at 22 h in comparison to 50 h with the higher inoculum concentration for xylose. These variations indicated that the yeast's biomass growth varies for the glucose, fructose, and xylose substrates. This is dependent on the yeast's ability to metabolize those sugars, as well as resource competition and the fermentation conditions (pH, aeration, temperature) [44]. The present study's result regarding the suitability of a higher inoculum concentration to reach the maximum yeast biomass concentration with a glucose substrate faster is in agreement with the literature [45]. Higher inoculum concentrations generally take less time to achieve optimal growth up to a certain limit; after that, the activity of the microbes reduces because of limited nutrient availability and competition [46].

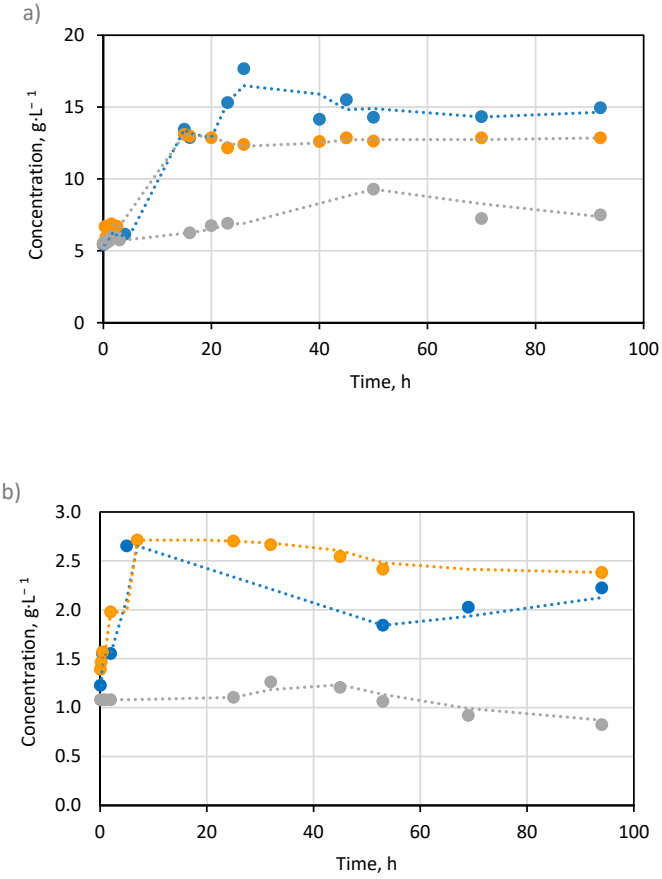

Figure 2. Variation in yeast concentration during the fermentation process in the case of a medium with fructose (●) glucose (●) and xylose (●), (**a**) at a high initial concentration (5.0 g·L^{-1}) of inoculum, and (**b**) at low initial concentration (1.1 g·L^{-1}) of inoculum.

Table 1 shows the maximum specific growth rate (μ_m) and biomass volumetric productivity (P_b) for the *H. polymorpha* yeast used for the sugar fermentation with different inoculum doses. The μ_m of the *H. polymorpha* yeast varied for fructose (0.121 h^{-1}), glucose (0.140 h^{-1}), and xylose (0.003 h^{-1}) at a higher inoculum concentration, while 0.159 h^{-1}, 0.175 h^{-1}, and 0.009 h^{-1} were achieved at the lower inoculum concentration, respectively. This indicated that the μ_m of yeast was relatively higher with glucose, followed by fructose and xylose, irrespective of the inoculum concentrations. Furthermore, the μ_m of yeast was comparatively higher with a lower inoculum concentration. In agreement with this finding, Mian et al. (1971) also observed that with an inferior amount of inoculum, the yeast (*Candida utilis*) exhibited a higher growth rate [47]. In comparison to the present values, earlier studies reported μ_m values of 0.16 h^{-1} to 0.42 h^{-1} for *H. polymorpha* grown

in glucose [48], whereas the μ_m values for *H. polymorpha* grown in xylose were 0.3 h^{-1} [49], 0.4 h^{-1} [40], and 0.04 h^{-1} [35].

Table 1. Maximum specific growth rate (μ_m), and biomass volumetric productivity (P_b) for the *H. polymorpha* yeast grown on fructose, glucose, and xylose.

Parameters	Units	Inoculum ($g \cdot L^{-1}$)	Fructose	Glucose	Xylose
μ_m	h^{-1}	1.1	0.159	0.175	0.009
μ_m	h^{-1}	5.0	0.121	0.140	0.003
P_b	$g \cdot L^{-1} h^{-1}$	1.1	0.270	0.185	0.001
P_b	$g \cdot L^{-1} h^{-1}$	5.0	0.473	0.483	0.069

The P_b was estimated based on the growth phase of the yeast. In cases with a higher inoculum concentration, the growth phases for fructose, glucose, and xylose were up to 26 h, 15 h, and 50 h. However, for the lower inoculum concentrations, the growth phases were 5 h, 6 h, and 25 h for the fructose, glucose, and xylose. In cases with higher inoculum concentrations, the P_b values were 0.483 $g \cdot L^{-1} h^{-1}$, 0.473 $g \cdot L^{-1} h^{-1}$, and 0.069 $g \cdot L^{-1} h^{-1}$ for glucose, fructose, and xylose, respectively, whereas with the lower inoculum concentration, the P_b values reduced to 0.270 $g \cdot L^{-1} h^{-1}$ (fructose), 0.185 $g \cdot L^{-1} h^{-1}$ (glucose), and 0.001 $g \cdot L^{-1} h^{-1}$ (xylose). In agreement with the present result, Escalante et al. (1990) reported a P_b of 0.18 $g \cdot L^{-1} h^{-1}$ for glucose fermentation at 45 °C and pH 4.8 [50]. Increasing the inoculum concentration from 1.1 $g \cdot L^{-1}$ to 5.0 $g \cdot L^{-1}$ elevated the P_b despite the use of different sugars. Glucose was found to be a more suitable substrate for obtaining the highest P_b of *H. polymorpha* yeast, and higher inoculum concentrations yielded better results. In contrast, the P_b of *H. polymorpha* yeast in xylose was comparatively lower than that in fructose and glucose.

3.4. Substrate Uptake

Table 2 presents the values of q_S^D and $Y_{X/S}^O$. The calculations of q_S^D were carried out on the basis of the fermentation time which showed the maximum concentration of yeast biomass. The q_S^D values were 0.310 $g \cdot g^{-1} h^{-1}$ (5 h, glucose), 0.129 $g \cdot g^{-1} h^{-1}$ (5 h, fructose), and 0.007 $g \cdot g^{-1} h^{-1}$ (50 h, xylose) with a higher inoculum concentration. The values of q_S^D decreased substantially with lower inoculum concentrations to 0.189 $g \cdot g^{-1} h^{-1}$ for glucose (5 h), whereas the q_S^D values increased to 0.579 $g \cdot g^{-1} h^{-1}$ and 0.022 $g \cdot g^{-1} h^{-1}$ for fructose (5 h) and xylose (32 h), respectively. A previous study by Sánchez et al. (1998) documented a similar value, i.e., a specific xylose uptake rate of 0.075 $g \cdot g^{-1} h^{-1}$ [40]. The $Y_{X/S}^O$ values in the case of the higher inoculum concentrations were 0.314 $g \cdot g^{-1}$, 0.264 $g \cdot g^{-1}$, and 0.126 $g \cdot g^{-1}$ for fructose, glucose, and xylose, which reduced to 0.307 $g \cdot g^{-1}$, 0.144 $g \cdot g^{-1}$, and 0.042 $g \cdot g^{-1}$ with the lower inoculum concentrations, respectively. This showed that a higher inoculum concentration was favorable for increasing $Y_{X/S}^O$ in case of xylose and glucose, whereas a change in the inoculum concentrations resulted in very narrow differences in the $Y_{X/S}^O$ values of glucose.

Table 2. Specific rate of substrate uptake (q_S^D), and biomass yield $\left(Y_{X/S}^O\right)$ for the *H. polymorpha* yeast grown on fructose, glucose, and xylose.

Parameters	Units	Inoculum ($g \cdot L^{-1}$)	Fructose	Glucose	Xylose
q_S^D	$g \cdot g^{-1} h^{-1}$	1.1	0.579 (5 h)	0.189 (7 h)	0.022 (32 h)
q_S^D	$g \cdot g^{-1} h^{-1}$	5.0	0.129 (5 h)	0.310 (5 h)	0.007 (50 h)
$Y_{X/S}^O$	$g \cdot g^{-1}$	1.1	0.307	0.144	0.042
$Y_{X/S}^O$	$g \cdot g^{-1}$	5.0	0.314	0.264	0.126

3.5. Ethanol Production

The *H. polymorpha* yeast produced ethanol through the fermentation of glucose, fructose, and xylose. Both the fermentation time and the inoculum concentration impacted the ethanol production (Figure 3). The sugar fermentation with a higher inoculum concentration showed the following trends: the ethanol concentration was found to be highest in fructose (16.75 $g \cdot L^{-1}$ at 23 h), followed by glucose (13.21 $g \cdot L^{-1}$ at 20 h), and then xylose (0.11 $g \cdot L^{-1}$ at 92 h). With lower inoculum concentrations, the maximum ethanol concentrations were observed to be higher for glucose (8.41 $g \cdot L^{-1}$ at 94 h), followed by fructose (9.80 $g \cdot L^{-1}$ at 94 h), and then xylose (0.87 $g \cdot L^{-1}$ at 45 h). Ryabova et al. (2003) documented 13.2 $g \cdot L^{-1}$ and 1.0 $g \cdot L^{-1}$ of ethanol production from glucose and xylose at 37 °C after 48 h of cultivation, respectively [36].

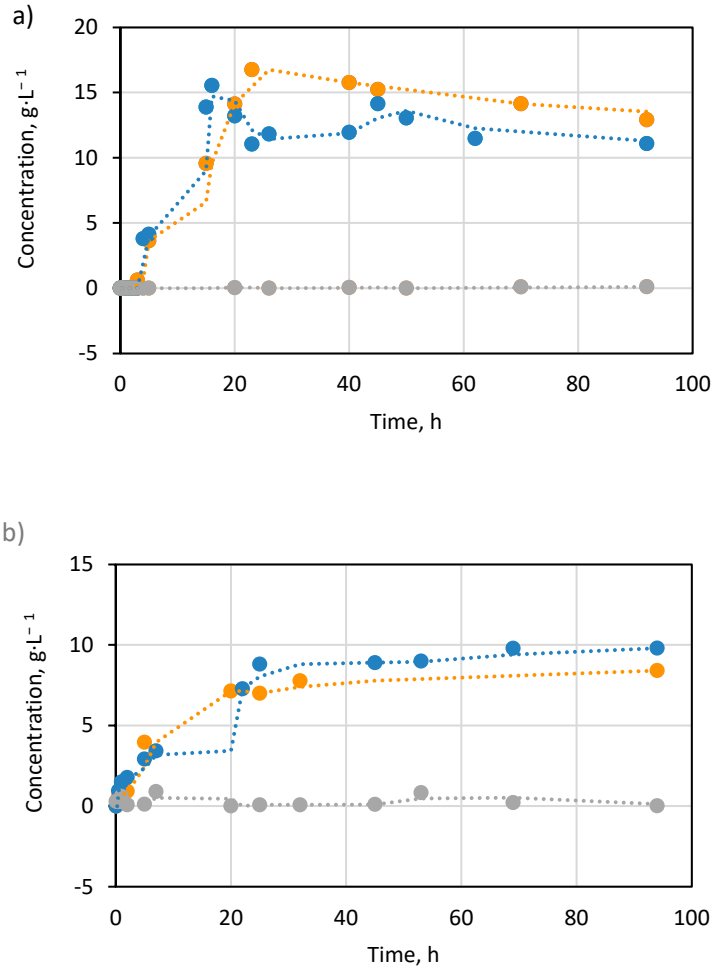

Figure 3. Variation in ethanol concentration during the fermentation process in the case of a medium with fructose (●), glucose (●), and xylose (●) (**a**) at high initial concentration (5.0 $g \cdot L^{-1}$) of inoculum, and (**b**) at low initial concentration (1.1 $g \cdot L^{-1}$) of inoculum.

The values of the specific production rates (q_{et}^{D}) and overall yields for ethanol (Y_{et}^{O}) are presented in Table 3. The higher inoculum concentrations were found to increase the specific rate of production and yield of ethanol. The q_{et}^{D} increased from 0.010 $g \cdot g^{-1}h^{-1}$ to 0.044 $g \cdot g^{-1}h^{-1}$ for fructose, and 0.010 $g \cdot g^{-1}h^{-1}$ to 0.053 $g \cdot g^{-1}h^{-1}$ for glucose. However, lowering the inoculum concentration increased the q_{et}^{D} for xylose from 0.01 $g \cdot g^{-1}h^{-1}$ to 0.028 $g \cdot g^{-1}h^{-1}$. The Y_{et}^{O} values were 0.322 $g \cdot g^{-1}$ (fructose), 0.258 $g \cdot g^{-1}$ (glucose), and 0.070 $g \cdot g^{-1}$ (xylose) with lower inoculum concentrations. An increase in the inoculum concentration produced a higher overall ethanol yield in the case of glucose (0.470 $g \cdot g^{-1}$) and fructose (0.434 $g \cdot g^{-1}$). However, a greater inoculum concentration did not appear to

have any noticeable impact on the overall ethanol yield (0.071 $g \cdot g^{-1}$) for xylose. Yamakawa et al. (2020) reported a similar ethanol yield of 0.42 ($g \cdot g^{-1}$) from *H. polymorpha* produced using glucose [35]. Other previous studies reported that xylose fermentation showed an overall ethanol yield of 0.30 $g \cdot g^{-1}$ for *H. polymorpha* [27], 0.31 $g \cdot g^{-1}$ for *K. marxianus* [51], and 0.44 $g \cdot g^{-1}$ *for P. stipitis* [52].

Table 3. Specific production rate and yields for ethanol from fructose, glucose and xylose with higher (5.0 $g \cdot L^{-1}$) and lower (1.1 $g \cdot L^{-1}$) inoculum concentrations of yeast.

Parameters	Units	Inoculum ($g \cdot L^{-1}$)	Fructose	Glucose	Xylose
q_{et}^{D}	$g \cdot g^{-1} h^{-1}$	1.1	0.010 (94 h)	0.010 (94 h)	0.028 (45 h)
q_{et}^{D}	$g \cdot g^{-1} h^{-1}$	5.0	0.044 (23 h)	0.053 (20 h)	0.010 (92 h)
Y_{et}^{O}	$g \cdot g^{-1}$	1.1	0.322	0.258	0.070
Y_{et}^{O}	$g \cdot g^{-1}$	5.0	0.434	0.470	0.071

Ethanol production from glucose, fructose, and xylose follows different pathways (Figure 4) [53]. The glucose and fructose fermentations produce ethanol through the Embden–Meyerhof–Parnas pathway (glycolysis reactions). This involves intermediary steps which cause successive transformations of glucose and fructose to 1,3 phosphate glycerate, pyruvate, acetaldehyde, and ethanol, whereas for the xylose fermentation, its initial conversion to xylulose is essential. The ethanol is produced from xylulose through the pentose phosphate pathway and glycolysis reactions. Furthermore, the fermentation of xylose using *H. Polymorpha* was reported to be dependent on the proper functioning of its peroxisomes, transadolases, and cytosolic transketolases. This dependency would be not necessary for glucose or fructose fermentation [52–55].

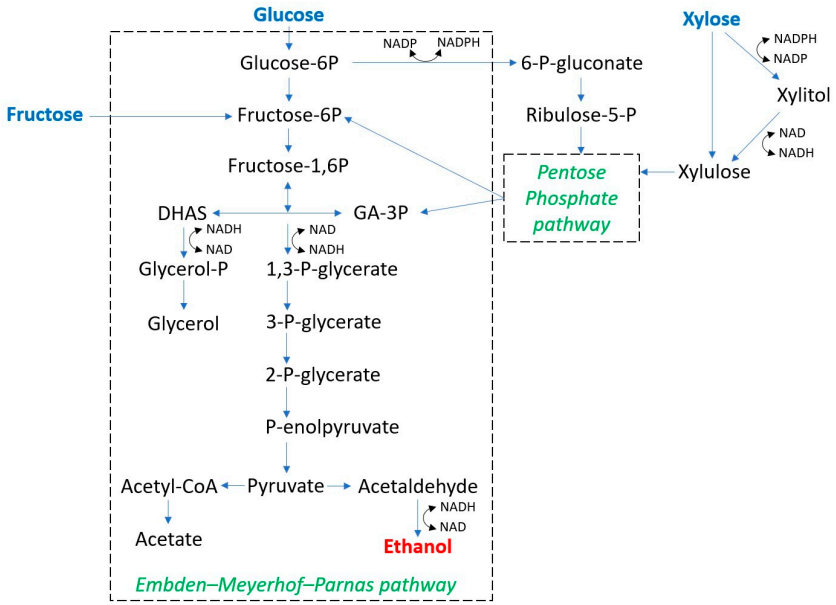

Figure 4. Scheme of different metabolic pathways for ethanol production from glucose, fructose, and xylose.

The cause of lower ethanol production from xylose substrates is not completely known. Sibirny (2023) suggested that the limited expression of regulatory enzymes (xylose reductase and xylitol dehydrogenase) for xylose metabolism could be a reason for the low ethanol yield from xylose fermentation. The xylose reductase enzyme utilizes NADPH and xylitol dehydrogenase utilizes NAD to oxidize xylitol to xylulose (which is essential for ethanol

production) through an oxidoreductive pathway. However, the insufficient re-oxidation of NADPH into NAD results in high xylitol production in place of ethanol [53].

4. Conclusions

This research delves into the potential of *H. polymorpha* for use in the high temperature fermentation of sugars (commonly found in lignocellulosic hydrolysates) to produce ethanol under limited aeration conditions. The inoculum concentrations, substrates (fructose, glucose, and xylose), and contact times impacted the growth and productivity of the yeast and the ethanol production. Fermentation time was found to be one of the deciding factors in achieving a higher yield of ethanol from glucose (25 h), fructose (26 h), and xylose (45 h). The utilization of 5.0 $g \cdot L^{-1}$ inoculum concentrations exhibited a greater overall yield of ethanol in the of case of glucose (0.47 $g \cdot g^{-1}$) and fructose (0.43 $g \cdot g^{-1}$), highly proximal to the theoretical value (0.51 $g \cdot g^{-1}$) of this parameter. Overall, the capability of thermotolerant *H. polymorpha* yeast to produce ethanol from the glucose, fructose, and xylose could be used for the high temperature (40–45 °C) fermentation of lignocellulosic hydrolysates (rich in aforementioned sugar) for ethanol production. The high-temperature fermentation using *H. polymorpha* yeast could also be beneficial from a technical and environmental point of view. This type of fermentation reduces the risk of contamination by other microorganisms that cannot survive at elevated temperatures, which ultimately improves the process' efficiency and limits the generation of fermentation process waste. Furthermore, *H. polymorpha* can grow and produce ethanol under limited aeration conditions. This further reduces the total cost of the fermentation process by minimizing the continuous aeration demand.

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Nomenclature

Symbols	Meaning
μ_m	Maximum specific growth rate
P_b	Biomass volumetric productivity
x	Cell concentration per volume
x_0	Cell concentration at the initial time
c	Empirical constant in the Equation (2)
q_S^D	Specific rate of substrate uptake

S	Substrate uptake at specific time
S_0	Substrate uptake at initial time
α and β	Constants measured using Equation (3)
$Y^O_{X/S}$	Overall biomass yield
X_p	Concentration of alcohol
a & b	Constants in the Equation (5)
q^D_{let}	Specific rate of ethanol production
Y^O_{et}	Overall ethanol yield

References

1. Reshmy, R.; Paulose, T.A.P.; Philip, E.; Thomas, D.; Madhavan, A.; Sirohi, R.; Binod, P.; Kumar Awasthi, M.; Pandey, A.; Sindhu, R. Updates on High Value Products from Cellulosic Biorefinery. *Fuel* **2022**, *308*, 122056. [CrossRef]
2. Claassen, P.A.M.; Lopez Contreras, A.M.; Sijtsma, L.; Weusthuis, R.A.; Van Lier, J.B.; Van Niel, E.W.J.; Stams, A.J.M.; De Vries, S.S. Utilisation of Biomass for the Supply of Energy Carriers. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 741–755. [CrossRef]
3. Hamelinck, C.N.; Faaij, A.P.C. Outlook for Advanced Biofuels. *Energy Policy* **2006**, *34*, 3268–3283. [CrossRef]
4. Rajesh Kumar, B.; Saravanan, S. Use of Higher Alcohol Biofuels in Diesel Engines: A Review. *Renew. Sustain. Energy Rev.* **2016**, *60*, 84–115. [CrossRef]
5. Mendiburu, A.Z.; Lauermann, C.H.; Hayashi, T.C.; Mariños, D.J.; Rodrigues da Costa, R.B.; Coronado, C.J.R.; Roberts, J.J.; de Carvalho, J.A. Ethanol as a Renewable Biofuel: Combustion Characteristics and Application in Engines. *Energy* **2022**, *257*, 124688. [CrossRef]
6. Limayem, A.; Ricke, S.C. Lignocellulosic Biomass for Bioethanol Production: Current Perspectives, Potential Issues and Future Prospects. *Prog. Energy Combust. Sci.* **2012**, *38*, 449–467. [CrossRef]
7. Reshmy, R.; Philip, E.; Unni, R.; Paul, S.A.; Sindhu, R.; Madhavan, A.; Sirohi, R.; Pandey, A.; Binod, P. Bioethanol—A Promising Alternative Fuel for Sustainable Future. In *Valorization of Biomass to Bioproducts: Organic Acids and Biofuels*; Elsevier: Amsterdam, The Netherlands, 2023.
8. David, A.J.; Abinandan, S.; Vaidyanathan, V.K.; Xu, C.C.; Krishnamurthi, T. A Critical Review on Current Status and Environmental Sustainability of Pre-Treatment Methods for Bioethanol Production from Lignocellulose Feedstocks. *3 Biotech* **2023**, *13*, 233. [CrossRef] [PubMed]
9. Melendez, J.R.; Mátyás, B.; Hena, S.; Lowy, D.A.; El Salous, A. Perspectives in the Production of Bioethanol: A Review of Sustainable Methods, Technologies, and Bioprocesses. *Renew. Sustain. Energy Rev.* **2022**, *160*, 112260. [CrossRef]
10. Peralta-Yahya, P.P.; Zhang, F.; Del Cardayre, S.B.; Keasling, J.D. Microbial Engineering for the Production of Advanced Biofuels. *Nature* **2012**, *488*, 320–328. [CrossRef]
11. Oh, Y.K.; Hwang, K.R.; Kim, C.; Kim, J.R.; Lee, J.S. Recent Developments and Key Barriers to Advanced Biofuels: A Short Review. *Bioresour. Technol.* **2018**, *257*, 320–333. [CrossRef]
12. Klein, B.C.; de Mesquita Sampaio, I.L.; Mantelatto, P.E.; Filho, R.M.; Bonomi, A. Beyond Ethanol, Sugar, and Electricity: A Critical Review of Product Diversification in Brazilian Sugarcane Mills. *Biofuels Bioprod. Biorefining* **2019**, *13*, 809–821. [CrossRef]
13. Kumar, A.K.; Sharma, S. Recent Updates on Different Methods of Pretreatment of Lignocellulosic Feedstocks: A Review. *Bioresour. Bioprocess.* **2017**, *4*, 7. [CrossRef] [PubMed]
14. Peinemann, J.C.; Pleissner, D. Continuous Pretreatment, Hydrolysis, and Fermentation of Organic Residues for the Production of Biochemicals. *Bioresour. Technol.* **2020**, *295*, 122256. [CrossRef]
15. Zabaniotou, A. Redesigning a Bioenergy Sector in EU in the Transition to Circular Waste-Based Bioeconomy-A Multidisciplinary Review. *J. Clean. Prod.* **2018**, *177*, 197–206. [CrossRef]
16. Conteratto, C.; Artuzo, F.D.; Benedetti Santos, O.I.; Talamini, E. Biorefinery: A Comprehensive Concept for the Sociotechnical Transition toward Bioeconomy. *Renew. Sustain. Energy Rev.* **2021**, *151*, 111527. [CrossRef]
17. Martins, M.; Sganzerla, W.G.; Forster-Carneiro, T.; Goldbeck, R. Recent Advances in Xylo-Oligosaccharides Production and Applications: A Comprehensive Review and Bibliometric Analysis. *Biocatal. Agric. Biotechnol.* **2023**, *47*, 102608. [CrossRef]
18. Weber, C.; Farwick, A.; Benisch, F.; Brat, D.; Dietz, H.; Subtil, T.; Boles, E. Trends and Challenges in the Microbial Production of Lignocellulosic Bioalcohol Fuels. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 1303–1315. [CrossRef] [PubMed]
19. Lee, J.W. *Advanced Biofuels and Bioproducts*, 1st ed.; Springer: New York, NY, USA, 2012.
20. Capilla, M.; Silvestre, C.; Valles, A.; Álvarez-Hornos, F.J.; San-Valero, P.; Gabaldón, C. The Influence of Sugar Composition and PH Regulation in Batch and Continuous Acetone–Butanol–Ethanol Fermentation. *Fermentation* **2022**, *8*, 226. [CrossRef]
21. Ruchala, J.; Kurylenko, O.O.; Dmytruk, K.V.; Sibirny, A.A. Construction of Advanced Producers of First- and Second-Generation Ethanol in *Saccharomyces Cerevisiae* and Selected Species of Non-Conventional Yeasts (*Scheffersomyces Stipitis*, *Ogataea Polymorpha*). *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 109–132. [CrossRef]
22. Lee, F.A. Alcoholic Fermentation. In *Basic Food Chemistry*; Springer: Dordrecht, The Netherlands, 1983.
23. Nanda, S.; Pattnaik, F.; Patra, B.R.; Kang, K.; Dalai, A.K. A Review of Liquid and Gaseous Biofuels from Advanced Microbial Fermentation Processes. *Fermentation* **2023**, *9*, 813. [CrossRef]
24. Kasavi, C.; Finore, I.; Lama, L.; Nicolaus, B.; Oliver, S.G.; Toksoy Oner, E.; Kirdar, B. Evaluation of *Industrial Saccharomyces Cerevisiae* Strains for Ethanol Production from Biomass. *Biomass Bioenergy* **2012**, *45*, 230–238. [CrossRef]

25. Lin, Y.; Zhang, W.; Li, C.; Sakakibara, K.; Tanaka, S.; Kong, H. Factors Affecting Ethanol Fermentation Using *Saccharomyces Cerevisiae* BY4742. *Biomass Bioenergy* **2014**, *47*, 395–401. [CrossRef]
26. Abdel-Banat, B.M.A.; Hoshida, H.; Ano, A.; Nonklang, S.; Akada, R. High-Temperature Fermentation: How Can Processes for Ethanol Production at High Temperatures Become Superior to the Traditional Process Using Mesophilic Yeast? *Appl. Microbiol. Biotechnol.* **2010**, *85*, 861–867. [CrossRef]
27. Kurylenko, O.O.; Ruchala, J.; Hryniv, O.B.; Abbas, C.A.; Dmytruk, K.V.; Sibirny, A.A. Metabolic Engineering and Classical Selection of the Methylotrophic Thermotolerant Yeast *Hansenula Polymorpha* for Improvement of High-Temperature Xylose Alcoholic Fermentation. *Microb. Cell Fact.* **2014**, *13*, 112. [CrossRef]
28. Mohd Azhar, S.H.; Abdulla, R.; Jambo, S.A.; Marbawi, H.; Gansau, J.A.; Mohd Faik, A.A.; Rodrigues, K.F. Yeasts in Sustainable Bioethanol Production: A Review. *Biochem. Biophys. Rep.* **2017**, *10*, 52–61. [CrossRef] [PubMed]
29. Cheng, J.J.; Timilsina, G.R. Status and Barriers of Advanced Biofuel Technologies: A Review. *Renew. Energy* **2011**, *36*, 3541–3549. [CrossRef]
30. Hoshida, H.; Akada, R. High-Temperature Bioethanol Fermentation by Conventional and Nonconventional Yeasts. In *Biotechnology of Yeasts and Filamentous Fungi*; Springer: Berlin/Heidelberg, Germany, 2017.
31. Gellissen, G.; Hollenberg, C.P. Hansenula. In *Enyclopedia of Food Microbiology*; Elsevier: Amsterdam, The Netherlands, 1999; pp. 976–982. [CrossRef]
32. Radecka, D.; Mukherjee, V.; Mateo, R.Q.; Stojiljkovic, M.; Foulquié-Moreno, M.R.; Thevelein, J.M. Looking beyond Saccharomyces: The Potential of Non-Conventional Yeast Species for Desirable Traits in Bioethanol Fermentation. *FEMS Yeast Res.* **2015**, *15*, fov053. [CrossRef] [PubMed]
33. Martínez, M.L.; Sánchez, S.; Bravo, V. Production of Xylitol and Ethanol by *Hansenula Polymorpha* from Hydrolysates of Sunflower Stalks with Phosphoric Acid. *Ind. Crops Prod.* **2012**, *40*, 160–166. [CrossRef]
34. Martínez-Cartas, M.L.; Olivares, M.I.; Sánchez, S. Production of Bioalcohols and Antioxidant Compounds by Acid Hydrolysis of Lignocellulosic Wastes and Fermentation of Hydrolysates with *Hansenula Polymorpha*. *Eng. Life Sci.* **2019**, *19*, 522–536. [CrossRef]
35. Yamakawa, C.K.; Kastell, L.; Mahler, M.R.; Martinez, J.L.; Mussatto, S.I. Exploiting New Biorefinery Models Using Non-Conventional Yeasts and Their Implications for Sustainability. *Bioresour. Technol.* **2020**, *309*, 123374. [CrossRef]
36. Ryabova, O.B.; Chmil, O.M.; Sibirny, A.A. Xylose and Cellobiose Fermentation to Ethanol by the Thermotolerant Methylotrophic Yeast *Hansenula polymorpha*. *FEMS Yeast Res.* **2003**, *4*, 157–164. [CrossRef]
37. du Preez, J.C. Process Parameters and Environmental Factors Affecting D-Xylose Fermentation by Yeasts. *Enzyme Microb. Technol.* **1994**, *16*, 944–956. [CrossRef]
38. Lindegren, C.C.; Nagai, S.; Nagai, H. Induction of Respiratory Deficiency in Yeast by Manganese, Copper, Cobalt and Nickel. *Nature* **1958**, *182*, 446–448. [CrossRef]
39. LSCI General Catalogue 2019. Languedoc Scientifique Company: Rivesaltes, France. Available online: https://lsci.fr/index.php/page-d-exemple/ (accessed on 13 May 2024).
40. Sánchez, S.; Bravo, V.; Castro, E.; Moya, A.J.; Camacho, F. The Production of Xylitol from D-Xylose by Fermentation with *Hansenula Polymorpha*. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 608–611. [CrossRef]
41. Bray, D. Critical Point Drying of Biological Specimens for Scanning Electron Microscopy. In *Supercritical Fluid. Methods and Protocols*; Humana Press: New York, NY, USA, 2003.
42. Manfrão-Netto, J.H.C.; Queiroz, E.B.; Rodrigues, K.A.; Coelho, C.M.; Paes, H.C.; Rech, E.L.; Parachin, N.S. Evaluation of Ogataea (Hansenula) Polymorpha for Hyaluronic Acid Production. *Microorganisms* **2021**, *9*, 312. [CrossRef]
43. Stöckmann, C.; Losen, M.; Dahlems, U.; Knocke, C.; Gellissen, G.; Büchs, J. Effect of Oxygen Supply on Passaging, Stabilising and Screening of Recombinant *Hansenula Polymorpha* Production Strains in Test Tube Cultures. *FEMS Yeast Res.* **2003**, *4*, 195–205. [CrossRef]
44. Ghoul, M.; Mitri, S. The Ecology and Evolution of Microbial Competition. *Trends Microbiol.* **2016**, *24*, 833–845. [CrossRef]
45. Ginovart, M.; Prats, C.; Portell, X.; Silbert, M. Exploring the Lag Phase and Growth Initiation of a Yeast Culture by Means of an Individual-Based Model. *Food Microbiol.* **2011**, *28*, 810–817. [CrossRef]
46. Kanwal, M.; Wattoo, A.G.; Khushnood, R.A.; Liaqat, A.; Iqbal, R.; Song, Z. Advancements and Challenges in Production of Biosurfactants. In *Applications of Next Generation Biosurfactants in the Food Sector*; Elsevier: Amsterdam, The Netherlands, 2022.
47. Mian, F.A.; Prokop, A.; Fencl, Z. Growth and Physiology of a Yeast Cultivated in Batch and Continuous Culture Systems. *Folia Microbiol.* **1971**, *16*, 249–259. [CrossRef] [PubMed]
48. Denenu, E.O.; Demain, A.L. Derivation of Aromatic Amino Acid Mutants from a Methanol-Utilizing Yeast, *Hansenula Polymorpha*. *Appl. Environ. Microbiol.* **1981**, *41*, 1088–1096. [CrossRef] [PubMed]
49. Feliu, J.A.; Mas, C.D.; González, G. SCP Production by *Hansenula Polymorpha* from Xylose. *Process Biochem.* **1990**, *25*, 136–140.
50. Escalante, J.; Caminal, G.; de Mas, C. Biomass Production by a Thermotolerant Yeast: *Hansenula Polymorpha*. *J. Chem. Technol. Biotechnol.* **1990**, *48*, 61–70. [CrossRef] [PubMed]
51. Wang, R.; Li, L.; Zhang, B.; Gao, X.; Wang, D.; Hong, J. Improved Xylose Fermentation of *Kluyveromyces Marxianus* at Elevated Temperature through Construction of a Xylose Isomerase Pathway. *J. Ind. Microbiol. Biotechnol.* **2013**, *40*, 841–854. [CrossRef] [PubMed]

52. Jeffries, T.W.; Grigoriev, I.V.; Grimwood, J.; Laplaza, J.M.; Aerts, A.; Salamov, A.; Schmutz, J.; Lindquist, E.; Dehal, P.; Shapiro, H.; et al. Genome Sequence of the Lignocellulose-Bioconverting and Xylose-Fermenting Yeast Pichia Stipitis. *Nat. Biotechnol.* **2007**, *25*, 319–326. [CrossRef]
53. Sibirny, A.A. Metabolic Engineering of Non-Conventional Yeasts for Construction of the Advanced Producers of Biofuels and High-Value Chemicals. *BBA Adv.* **2023**, *3*, 100071. [CrossRef]
54. Kurylenko, O.O.; Ruchala, J.; Vasylyshyn, R.V.; Stasyk, O.V.; Dmytruk, O.V.; Dmytruk, K.V.; Sibirny, A.A. Peroxisomes and Peroxisomal Transketolase and Transaldolase Enzymes Are Essential for Xylose Alcoholic Fermentation by the Methylotrophic Thermotolerant Yeast, Ogataea (Hansenula) Polymorpha. *Biotechnol. Biofuels* **2018**, *11*, 197. [CrossRef]
55. Jeffries, T.W.; Jin, Y.S. Metabolic Engineering for Improved Fermentation of Pentoses by Yeasts. *Appl. Microbiol. Biotechnol.* **2004**, *63*, 495–509. [CrossRef]