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Exploring Natural Fermented Foods as a Source for New Efficient Thermotolerant Yeasts for the Production of Second-Generation Bioethanol

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Abstract: Considering the cost-effectiveness of bioethanol production at high temperatures, there is an enduring need to find new thermotolerant ethanologenic yeasts. In this study, a total of eighteen thermotolerant yeasts were isolated from various natural fermented products in Morocco. Ethanol production using 50 g/L glucose or 50 g/L xylose as the sole carbon source revealed potential yeasts with high productivities and volumetric ethanol productivities at high temperatures. Based on molecular identification, the selected thermotolerant fermentative isolates were affiliated with *Pichia kudriavzevii*, *Kluyveromyces marxianus*, and *Kluyveromyces* sp. During the simultaneous saccharification and fermentation of lignocellulosic biomass at a high temperature (42 °C), the designated yeast *P. kudriavzevii* YSR7 produced an ethanol concentration of 22.36 g/L, 18.2 g/L and 6.34 g/L from 100 g/L barley straw (BS), chickpea straw (CS), and olive tree pruning (OTP), respectively. It also exhibited multi-stress tolerance, such as ethanol, acetic acid, and osmotic tolerance. Therefore, the yeast *P. kudriavzevii* YSR7 showed promising attributes for biorefinery-scale ethanol production in the future.

Keywords: lignocellulosic biomass; fermentation; bioethanol; thermotolerant yeasts; stress tolerance

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1. Introduction

In the current scenario, global warming and rising socio-environmental problems are forcing societies to look for alternative, green and sustainable biofuel products. Among them, bioethanol is considered one of the most potent liquid biofuels in the bioenergy industry. Reports from the Renewable Fuels Association (US) indicate that global bioethanol production increased from 13.12 billion gallons to 29.03 billion gallons from 2007 to 2019 [1,2]. The use of bioethanol is mainly linked to the transportation sector. It is usually blended with petroleum fuels because it enhances fuel efficiency due to its high octane rating, large flammability spectrum, increased vaporization temperatures, and most importantly, because it reduces greenhouse gas emissions [3–5].

Second generation bioethanol refers to bioethanol obtained from lignocellulosic biomass resources [6,7]. Several strategies are implicated in the bioethanol production process, such as simultaneous saccharification and fermentation (SSF), separate saccharification and fermentation (SHF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP). However, SSF remains the focus of many studies because it reduces processing tools, residence time, risk of contamination, and investment costs [8–10]. In this approach, cellulase enzymes and fermenting microorganism are mixed in at the same time in the same vessel with the pretreated lignocellulosic biomass, permitting ethanol production in a single step [11,12]. The main drawback of the SSF is the compromised

optimal temperature between enzymatic hydrolysis (45–50 °C) and fermentation (30 °C). In general, the temperature in the SSF process is set close to the optimal temperature for saccharification rather than that for fermentation [11]. Therefore, increasing the bioconversion yields depends on the conditions and on the use of thermotolerant microorganisms with high ethanol production and tolerance abilities.

Yeast species are most often employed in bioethanol fuel factories. They exhibit valuable characteristics such as high ethanol productivity, high ethanol tolerance, fermentation of a wide variety of sugars, and growth in low-cost media [4]. Several researchers have characterized *P. kudriavzevii*, *Kluyveromyces marxianus*, *Candida tropicalis*, and some strains of *Saccharomyces cerevisiae* as thermotolerant fermentative strains [13–16]. Nevertheless, defects associated with osmotic stress and inhibitors generated after pretreatment (e.g., weak acids, furan aldehydes, and phenolic compounds) or during fermentation (e.g., ethanol) still limit ethanol production and the growth performance of some thermoethalogenic yeasts [17]. Another reported challenge is the fermentation of sugar C-5 (i.e., xylose), the second most abundant sugar in lignocellulosic biomass [18]. Yeasts generate lower levels of bioethanol from sugar C-5 compared to C-6 (i.e., glucose) [19]. It is therefore necessary to explore new efficient thermo-ethalogenic yeasts from potential biotopes able to ferment sugars C-5 and C-6. Natural fermented foods and beverages are prepared through a spontaneous and uncontrolled fermentation of complex microbial diversity [20]. For example, yeast cells retrieved in traditional sourdoughs are known to tolerate a wide range of stress conditions such as nutrient deficiency and complexity, acidic pH, temperature fluctuations, and high osmotic stress [21].

The objective of this study was to isolate and select potential thermotolerant yeasts from natural fermented products in Morocco and to evaluate their ability to produce bioethanol at high temperatures (42 °C) using glucose, xylose, and alkali treated lignocellulosic feedstocks as substrates. In addition, an evaluation of the tolerance to certain stress conditions encountered during ethanol fermentation was performed.

2. Materials and Methods

2.1. Samples Collection and Yeasts Isolation

Twelve sourdoughs (spontaneous fermented dough), Four Raib (spontaneous fermented yogurt), four Leben (spontaneous fermented milk), and three Smen (spontaneous fermented butter) were collected from various locations in the region of Fez, Morocco. All samples were prepared in a traditional manner without the addition of any commercial fermenting microorganisms. Ten grams of each sample were weighed, aseptically suspended in 90 mL of sterile physiological water and vortexed for 15 min at room temperature. Serial dilutions were made and 0.1 mL of each dilution was spread into a YPD agar (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of glucose, 15 g/L of agar) supplemented with chloramphenicol (100 µg/mL). YPD plates were incubated for 48 h at 30 °C. Pure colonies were peaked with a conventional streaking technique on YPD agar and the stock cultures of the isolates were maintained in glycerol broth (30%) at −80 °C for long-term storage.

2.2. Screening and Characterization of Yeasts Isolates

Thermotolerant yeast isolates were selected based on their growth performance on YPD agar plates incubated at 37, 40, 45 and 47 °C for 48 h. To screen for fermentative yeasts, the thermotolerant isolates were grown in test tubes containing 20 mL of YPD broth supplemented with 31.56 g/L ethanol and Durham tubes. Incubation was carried out at 40 °C for 48 h with stirring at 150 rpm. The objective was to evaluate the capacity of yeast isolates on accumulating carbon dioxide (CO_2) gas bubbles in Durham tubes (Sigma-Aldrich, Saint-Louis, MO, USA). Regarding the sugar utilization profile, the pure cultures of yeasts were suspended in YP broth (10 g/L of yeast extract, 20 g/L of peptone) supplemented with 20 g/L of the following sugars: glucose, galactose, xylose, arabinose, maltose, and sucrose. Incubation was performed at 40 °C for 48 h, and assimilation was evaluated by analyzing the visual growth of isolates.

2.3. Assessment of Bioethanol Production at High Temperatures Using Glucose and Xylose as Substrates

The ability of yeast isolates to produce ethanol at elevated temperatures was evaluated using glucose or xylose as the sole carbon source. Selected isolates were grown to the exponential phase in 20 mL of YPD or YPX liquid medium and the cultures were adjusted to a concentration of OD_{600} = 1. A volume of 2 mL of each culture was inoculated onto 50 mL of fermentation medium (pH 5) containing sugar at 50 g/L, yeast extract 5 g/L, peptone 5 g/L, K_2HPO_4 1 g/L, and $MgSO_4$ 1 g/L, then incubated at 40, 42, and 45 °C for 48 h in a rotatory incubator shaker at 150 rpm. The concentration of ethanol produced was estimated spectrophotometrically using the potassium dichromate method [22]. Experiments were performed in duplicate.

2.4. Molecular Identification of the Selected Thermotolerant Fermentative Yeasts

The high-performing thermotolerant fermentative isolates were identified based on the amplification of the 5.8S-internal transcribed spacer (ITS) region using the polymerase chain reaction (PCR) method. Genomic DNA was isolated from yeasts cells according to the method described by Harju et al. [23] and subjected to amplification using the specific primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′). The PCR reaction mix (50 µL) contained the following: Taq buffer (×1), MgCl2 (15 mM), dNTP (0.2 mM), Taq polymerase (0.04 U/µL), primers ITS1 and ITS4 (0.25 µM, each), pure water and DNA (1 µL). The amplification reaction was carried out for 30 cycles of 45 s at 94 °C, 60 s at 55 °C and 60 s at 72 °C, with an initial denaturation step of 5 min at 94 °C and a final step of extension for 10 min at 72 °C. The PCR products obtained were sequenced using Applied Biosystems® 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and compared to the sequences published in the GenBank NCBI database using BLASTN homology searches.

2.5. Bioethanol Production from Pretreated Lignocellulosic Biomass through SSF Approach

2.5.1. Alkaline Pretreatment of Lignocellulosic Biomass

Barley straw (BS), chickpea straw (CS), and olive tree pruning (OTP) samples were collected from a farm located in Fez, Morocco. The raw biomass was washed, dried, and ground with a knife mill to a particle size of less than 1 mm. For pretreatment, 100 g/L of each biomass was soaked in a 4% sodium hydroxide (NaOH) solution at 100 °C for 60 min. The solid fraction was collected by filtration and washed with distilled water until neutralization of pH. Then, the pretreated biomass was dried at 50 °C and sealed in plastic bags until use.

2.5.2. Simultaneous Saccharification and Fermentation

The selected yeast strain was tested for the production of second generation bioethanol by adopting the SSF approach. First, pure colonies of the yeast were inoculated into 100 mL of YPD broth at 35 °C and 150 rpm. After 24 h of incubation, cell pellets were harvested by centrifugation for 10 min at 12,298× *g*, washed with sterile distilled water, and suspended in 0.9% of sodium chloride solution. Yeast inoculum was adjusted to a concentration of OD_{600} = 1. SSF assays were carried out in a 100 mL sodium acetate buffer (50 mM, pH 5) in which the following were suspended: 100 g/L of alkali pretreated biomass, 5 g/L of yeast extract, 5 g/L peptone, 1 g/L of K_2HPO_4, and 1 g/L of $MgSO_4$. The fermentation medium was sterilized for 15 min at 120 °C. Then, commercial cellulosic enzymes (*Aspergillus niger* cellulose, Sigma-Aldrich, Saint-Louis, MO, USA) with loadings of 30 FPU/g of biomass and 10% of yeast inoculum were added aseptically. Incubation was performed at 42 °C with 150 rpm. Samples were withdrawn after 24, 48, and 72 h of fermentation.

2.6. Evaluation of Growth under Fermentative Stress Conditions

The tolerance of the selected yeast to various stress conditions was tested according to the protocol described by Chamnipa et al. [13] with modifications. First, the strain was grown overnight in YPD broth at 35 °C with shaking at 150 rpm. After incubation, the culture was recovered by centrifugation for 10 min at 12,298× *g*, washed twice with sterile distilled water, and adjusted to a concentration of OD_{600} = 0.1. Serial dilutions were made and 10 µL was spotted onto the appropriate agar medium. For ethanol stress, cells were grown in YPD agar supplemented with ethanol concentrations of 47.34, 63.12, 78.9, 94.68 and 110.46 g/L. For Acetic acid stress, cells were grown in YPD agar supplemented with acetic acid concentrations of 1, 3, 5, 7 and 9 g/L. For osmotic stress, cells were grown in YPD agar with glucose concentrations of 100, 200, 300, 400 and 500 g/L. The plates were incubated at 35 °C for 48 h. Tolerance to the above stressors was assessed by analyzing the visual growth of colonies.

3. Results and Discussion

3.1. Isolation and Evaluation of Thermotolerance, Sugars Assimilation and Fermentative Capacity of Yeasts

The use of efficient thermotolerant ethanologenic yeasts is one of the keys to successfully overcoming the challenges associated with ethanol production at high temperatures (<40 °C). In this study, a total of fifty yeast isolates were obtained from the different samples analyzed. Thirty-two were isolated from Sourdoughs (YSR1-YSR32), eight from Raib (YRB1-YRB8), six from Leben (YLB1-YLB6), and four from Smen (YSM1-YSM4). They were all investigated for their thermotolerant character at temperatures of 37, 40, 45 and 47 °C after 48 h of incubation in YPD agar. As shown in Table 1, thirty eight isolates grew well at 37 °C, while eighteen isolates grew well at 40 °C, ten isolates showed good growth at 45 °C, and finally, four isolates, specifically YSR32, YRB4, YLB2, YLB4, resisted the highest temperature of 47 °C and showed moderate growth. According to the definition given by Sree et al. [24], eighteen isolates were classified in this study as thermotolerant yeasts because they grew at a temperature of 40 °C. To screen for fermentative yeasts, the selected thermotolerant isolates were seeded in 20 mL of YPD broth containing Durham tubes and 31.56 g/L ethanol. The results demonstrated (Supplementary Materials) that all of them were able to accumulate important concentrations of CO_2 gas in Durham tubes which indicated their fermentation capacity. Several researchers have demonstrated that natural fermented foods and beverages provide an attractive niche for the isolation of robust thermotolerant ethanogenic yeasts. For example, Talukder et al. [25] reported the isolation of thermotolerant yeasts from a variety of natural fermented sources collected in Bangladesh. They found eighteen yeasts that were able to produce ethanol effectively at high temperatures. Choi et al. [26] also described the isolation of thermotolerant yeasts from nuruk, a traditional Korean fermentation starter. They found a yeast, identified as *P. kudriavzevii* MBY1358, that showed rapid growth and high ethanol productivity at elevated temperatures of 44 °C.

The enzymatic hydrolysis of lignocellulosic biomass mainly generates glucose from the cellulosic fraction and xylose from the hemicellulosic fraction, as well as arabinose, galactose, mannose, and cellobiose [27]. Hence, to further improve ethanol yields, it is recommended to use yeasts capable of assimilating and fermenting a broad spectrum of sugars. As displayed in Table 1, the majority of the isolates metabolized glucose, galactose, maltose and sucrose, unlike sugar C-5, of which only six isolates (YSR7, YSR29, YSR32, YRB8, YLB2, and YLB4) assimilated effectively, as well as xylose and one isolate arabinose (YLB4).

Table 1. Temperature and sugar assimilation profiles of the isolated yeasts.

Yeasts Isolates	Temperatures (°C)				Sugars Assimilation					
					Hexose Sugars		Pentose Sugars		Disaccharides	
	37	40	45	47	Glucose	Galactose	Xylose	Arabinose	Maltose	Sucrose
YSR1	++	++	++	−	++	++	+	+	++	++
YSR2	++	−	−	−	++	++	−	−	++	++
YSR3	++	+	−	−	++	++	−	−	++	++
YSR4	+	−	−	−	++	++	−	−	++	++
YSR5	++	+	−	−	++	++	−	−	++	++
YSR6	++	+	−	−	++	+	−	−	++	++
YSR7	++	++	++	−	++	++	++	+	−	++
YSR8	++	+	−	−	++	−	−	−	++	++
YSR9	++	++	++	−	++	−	+	−	−	++
YSR10	+	+	−	−	++	++	−	−	++	++
YSR11	−	−	−	−	++	++	−	−	++	++
YSR12	++	++	+	−	++	++	+	+	+	++
YSR13	++	−	−	−	++	++	−	−	++	++
YSR14	++	+	−	−	++	+	−	−	++	++
YSR15	++	++	+	−	++	++	+	−	−	++
YSR16	++	+	−	−	++	+	−	−	++	++
YSR17	++	−	−	−	++	−	−	−	++	++
YSR18	++	+	−	−	++	+	−	−	++	++
YSR19	++	+	−	−	++	++	−	−	++	++
YSR20	++	++	+	−	++	++	−	−	++	++
YSR21	+	+	−	−	++	++	−	−	++	++
YSR22	++	−	−	−	++	++	−	−	++	++
YSR23	++	++	+	−	++	++	−	−	++	++
YSR24	++	+	−	−	++	−	−	−	++	++
YSR25	++	++	+	−	++	++	−	−	++	++
YSR26	+	−	−	−	++	++	−	−	++	++
YSR27	+	−	−	−	++	++	−	−	++	++
YSR28	+	+	−	−	++	++	−	−	++	++
YSR29	++	++	++	−	++	++	++	+	++	++
YSR30	++	+	−	−	++	++	−	−	++	++
YSR31	++	+	−	−	++	++	−	−	++	++
YSR32	++	++	++	+	++	++	++	+	−	++
YRB1	++	++	++	−	++	++	+	−	++	++
YRB2	+	+	−	−	++	++	−	−	−	++
YRB3	++	+	−	−	++	++	−	−	−	++
YRB4	++	++	++	+	++	++	−	−	−	++
YRB5	++	+	−	−	++	++	−	−	++	++
YRB6	++	+	−	−	++	++	−	−	++	++
YRB7	++	++	++	−	++	++	+	−	++	++
YRB8	++	++	+	−	++	++	++	−	++	++
YLB1	+	−	−	−	++	+	−	−	++	++
YLB2	++	++	++	+	++	++	++	+	++	++
YLB3	++	+	−	−	++	++	−	−	++	++
YLB4	++	++	++	+	++	++	++	++	++	++
YLB5	++	+	−	−	++	++	−	−	++	++
YLB6	++	++	+	−	++	++	−	−	++	++
YSM1	+	+	−	−	++	−	−	−	+	++
YSM2	+	−	−	−	++	++	−	−	+	++
YSM3	++	++	+	−	++	++	−	−	−	++
YSM4	+	+	−	−	++	+	−	−	−	++

(++): Good growth; (+): moderate growth; (−): no growth.

3.2. Selection and Molecular Identification of Efficient Thermo-Ethalogenic Yeasts

To confirm the ability of the eighteen selected yeast isolates to produce ethanol at high temperatures (40, 42, and 45 °C), fermentation kinetics were evaluated using glucose or

xylose as substrates. Table 2 presents the results of fermentation using 50 g/L glucose. At 40 °C, ethanol productivity ranged from 7.13 to 18.19 g/L, and volumetric ethanol productivity ranged from 0.15 to 0.38 g/L h. At 42 °C, ethanol productivity ranged from 5.39 to 18.54 g/L, and volumetric ethanol productivity ranged from 0.11 to 0.39 g/L h. At the highest temperature of 45 °C, the kinetics parameters significantly dropped with values ranging from 4.16 to 13.79 g/L, and from 0.09 to 0.29 g/L h for ethanol productivity and volumetric ethanol productivity, respectively.

Table 2. Ethanol production potential of the selected yeast isolates at high temperatures using 50 g/L glucose as substrate.

Isolates	Temperatures (°C)					
	40		42		45	
	P (g/L)	Qp (g/L h)	P (g/L)	Qp (g/L h)	P (g/L)	Qp (g/L h)
YSR1	15.4 ± 0.02	0.32 ± 0.00	15.41 ± 0.13	0.32 ± 0.00	10.83 ± 0.18	0.22 ± 0.00
YSR7	18.19 ± 0.56	0.38 ± 0.01	18.54 ± 0.21	0.39 ± 0.00	13.79 ± 0.05	0.29 ± 0.00
YSR9	14.53 ± 0.13	0.30 ± 0.00	14.46 ± 0.02	0.30 ± 0.00	10.16 ± 0.19	0.21 ± 0.00
YSR12	10.47 ± 0.09	0.21 ± 0.00	10.56 ± 0.10	0.22 ± 0.00	6.44 ± 0.11	0.13 ± 0.00
YSR15	12.82 ± 0.02	0.27 ± 0.00	9.20 ± 0.80	0.19 ± 0.02	6.95 ± 0.82	0.14 ± 0.02
YSR20	7.83 ± 0.37	0.16 ± 0.01	8.58 ± 0.03	0.18 ± 0.00	5.34 ± 0.01	0.11 ± 0.00
YSR23	14.07 ± 0.02	0.29 ± 0.00	13.25 ± 0.10	0.27 ± 0.00	9.55 ± 0.05	0.20 ± 0.00
YSR25	9.66 ± 0.15	0.20 ± 0.00	9.3 ± 0.13	0.19 ± 0.00	6.50 ± 0.13	0.14 ± 0.00
YSR29	15.46 ± 0.07	0.32 ± 0.00	15.68 ± 0.10	0.33 ± 0.00	11.45 ± 0.25	0.24 ± 0.01
YSR32	16.49 ± 0.11	0.34 ± 0.00	14.83 ± 0.19	0.31 ± 0.00	12.04 ± 0.62	0.25 ± 0.01
YRB1	15.1 ± 10.18	0.31 ± 0.00	14.63 ± 0.03	0.30 ± 0.00	10.34 ± 0.20	0.22 ± 0.00
YRB4	13.22 ± 0.15	0.28 ± 0.00	14.52 ± 0.08	0.30 ± 0.00	10.18 ± 0.20	0.21 ± 0.00
YRB7	14.58 ± 0.25	0.30 ± 0.01	13.88 ± 0.03	0.29 ± 0.00	10.15 ± 0.15	0.21 ± 0.00
YRB8	11.75 ± 0.13	0.24 ± 0.00	11.45 ± 0.17	0.24 ± 0.00	8.82 ± 0.02	0.18 ± 0.00
YLB2	13.86 ± 0.15	0.29 ± 0.00	13.43 ± 0.01	0.28 ± 0.00	11.78 ± 0.12	0.25 ± 0.00
YLB4	15.10 ± 0.13	0.31 ± 0.00	13.33 ± 0.92	0.28 ± 0.02	10.96 ± 0.40	0.23 ± 0.01
YLB6	14.09 ± 0.17	0.29 ± 0.00	13.37 ± 0.10	0.28 ± 0.00	10.45 ± 0.53	0.22 ± 0.01
YSM3	7.13 ± 0.60	0.15 ± 0.01	5.39 ± 0.17	0.11 ± 0.00	4.16 ± 0.12	0.09 ± 0.00

P (g/L): Ethanol productivity; Qp (g/L h): Volumetric ethanol productivity.

The six xylose-assimilating isolates were assessed for ethanol fermentation using 50 g/L of this sugar as substrate (Table 3). At 40 °C, ethanol productivity ranged from 2.95 to 12.34 g/L, and volumetric ethanol productivity ranged from 0.06 to 0.26 g/L h. At 42 °C, ethanol productivity ranged from 1.24 to 10.72 g/L, and volumetric ethanol productivity ranged from 0.03 to 0.22 g/L h. At 45 °C, only four isolates maintained ethanol production with ethanol productivity and volumetric ethanol productivity ranging from 2.97 to 4.48 g/L, and from 0.06 to 0.09 g/L h, respectively. Yeast isolates YSR29 and YRB8 were unable to produce ethanol from xylose at 45 °C.

Table 3. Ethanol production potential of the selected yeast isolates at high temperatures using 50 g/L xylose as substrate.

Isolates	Temperatures (°C)					
	40		42		45	
	P (g/L)	Qp (g/L h)	P (g/L)	Qp (g/L h)	P (g/L)	Qp (g/L h)
YSR7	12.34 ± 0.45	0.26 ± 0.01	8.10 ± 0.37	0.16 ± 0.01	3.80 ± 0.33	0.08 ± 0.01
YSR29	2.95 ± 0.14	0.06 ± 0.00	1.24 ± 0.37	0.03 ± 0.01	0 ± 0.00	0 ± 0.00
YSR32	12.33 ± 0.30	0.26 ± 0.01	10.72 ± 0.51	0.22 ± 0.01	4.48 ± 0.04	0.09 ± 0.00
YRB8	5.57 ± 0.30	0.12 ± 0.01	2.06 ± 0.25	0.04 ± 0.01	0 ± 0.00	0 ± 0.00
YLB2	5.55 ± 0.42	0.12 ± 0.01	5.56 ± 0.15	0.12 ± 0.00	3.16 ± 0.06	0.07 ± 0.00
YLB4	6.42 ± 0.23	0.13 ± 0.00	5.13 ± 0.14	0.11 ± 0.00	2.97 ± 0.26	0.06 ± 0.01

P (g/L): Ethanol productivity; Qp (g/L h): Volumetric ethanol productivity.

Yeast isolates YSR7, YSR32, YSR29, YRB1, and YSR1 were chosen for molecular identification by sequencing the 5.8S-ITS regions based on their performance in ethanol production from glucose and xylose. The analysis of homology percentages on the Gene Bank BLASTN database showed that the isolates YSR7, YSR32, YSR29, YRB1, and YSR1 were closely related to *P. kudriavzevii* (MH545928), *K. marxianus* (NR_111251), *K. marxianus* (KY103793), *K. marxianus* (NR_111251), and *K. marxianus* (MW284516) with an homology percentage of 99.59, 99.12, 99.38, 99.70, 99.32%, respectively. As noticed, four of the strains identified belonged to the genus *Kluyveromyces*, indicating that this genus is prevalent in this biotype.

The yeast *P. kudriavzevii* YSR7 gave the maximum results in terms of ethanol productivity and ethanol volumetric productivity from 50 g/L glucose among all the isolated yeasts. Choudhary et al. [15] reported that two strains of *P. kudriavazevii* JRC2P and JRC4 produced ethanol concentrations of 16.6 and 18.6 g/L from 50 g/L glucose at 40 °C. In the same context, using a high concentration of the substrate, Chamnipa et al. [13] described that six yeast strains identified as *P. kudriavzevii* provided ethanol titers in the range of 37.56–70.51 g/L at 40 °C and 36.27–45.10 g/L at 45 °C from 160 g/L glucose. Likewise, Techaparin et al. [17] stated that *P. kudriavzevii* KKU-LA28 and KKU-TH199 produced ethanol concentrations of 44.68 and 43.27 g/L from 200 g/L glucose at 40 °C. For xylose fermentation, the ethanol yields obtained were clearly lower than those from glucose. Xylose fermentation is a complex metabolic process. It has been said that one of the reasons for the low levels of ethanol from this sugar is that elevated temperatures and low oxygen availability considerably reduce the rate of conversion of xylose to ethanol, as they decrease the concentrations of ATP required for the functioning of xylose transporters in xylose-fermenting yeasts [28]. Yeasts that ferment xylose have been isolated from different habitats such as decaying wood, tree exudates, wood-boring insects, soil, rotten fruits, and flowers [29–32]. However, to the best of our knowledge, this is the first report describing the isolation of potential xylose fermenting yeasts from natural sourdoughs. The strains *K. marxianus* YSR32 and *P. kudriavzevii* YSR7 displayed high concentrations of ethanol under anaerobic conditions; there was no significant difference between the two strains at 40 °C. Rodrussamee et al. [33] reported that the thermotolerant yeast *K. marxianus* DMKU3-1042 produced about 2.6, 2.2 and 0.96 g/L ethanol at 30 °C, 40 and 45 °C from 20 g/L xylose, respectively. On the other hand, Nweze et al. [34] reported that ethanol productivities were 6.9 g/L and 6.3 g/L from 30 g/L xylose at 42 °C after 96 h of incubation for *P. kudriavzevii* Pi131 and *P. kudriavzevii* Pa27, respectively.

3.3. Simultaneous Saccharification and Fermentation Using Alkali Treated Biomasses

The yeast *P. kudriavzevii* YSR7 statistically exhibited the best performance in the bioconversion of glucose to ethanol. Therefore, this strain was selected for SSF using alkali-treated BS, CS, and OTP at 42 °C for 72 h. As shown in Figure 1, the maximum ethanol concentration was obtained from BS biomass with 22.36 g/L, followed by CS with 18.2 g/L, and finally by OTP with 6.34 g/L. Strains of *P. kudriavzevii* have been previously implicated in the SSF process. Oberoi et al. [35] found an ethanol concentration of 24.25 g/L from sodium hydroxide-treated rice straw using the *P. kudriavzevii* HOP-1 strain. In another study, Yuan et al. [36] obtained 33.4 g/L ethanol from acid-impregnated steam explosion-treated rice straw using the *P. kudriavzevii* SI strain. The authors performed SSF with a high solid load of 200 g/L and with low inhibitor concentrations (furfural 0.19 g/L and acetic acid 0.95 g/L), which may favor their ethanol production results. In this study, *P. kudriavzevii* YSR7 generated the lowest ethanol titers from OTP compared to other agricultural residues tested. This may be explained by the fact that this type of biomass has a very recalcitrant structure and requires harsh pretreatment conditions to enhance the accessibility of cellulolytic enzymes to carbohydrates. There are few reports dealing with SSF using hard biomass and the species of *P. kudriavzevii*. For example, Akita et al. [37] stated that *P. kudriavzevii* NBRC1279 and NBRC1664 produced 21.6 g/L and 21.3 g/L ethanol, respectively, from Japanese eucalyptus at 30 °C for 144 h.

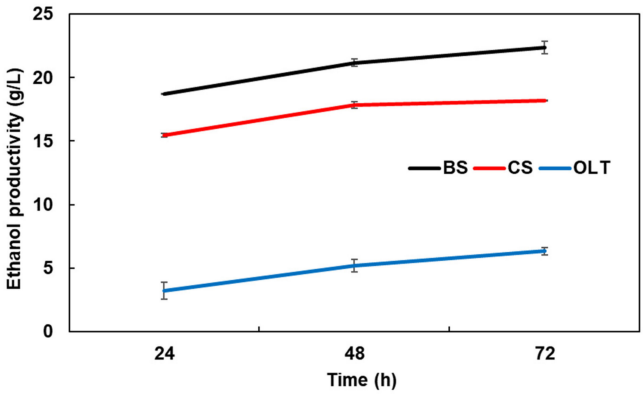

Figure 1. Ethanol production profile (g/L) of the selected yeast *P. kudriavzevii* YSR7 during SSF at 42 °C of alkali- treated BS, CS, and OTP biomasses.

3.4. Evaluation of Growth under Fermentative Stress Conditions

Gaining high ethanol titers from lignocellulosic biomass remains a technical challenge, as yeasts are confronted with several stressful conditions during fermentation such as heat, oxidative stress, osmotic pressure, inhibitory by-products, and an acidic pH [38]. These stress factors are well known to negatively affect cell growth and metabolism. For this reason, the growth response of *P. kudriavzevii* YSR7 over different stress conditions was investigated.

Ethanol levels above 78.9 g/L significantly decrease the growth rate of yeasts, destabilize the cell wall structure, alter the transport system involved in glucose uptake and inhibit the function of certain enzymes [39–45]. As illustrated in Figure 2a, *P. kudriavzevii* YSR7 was able to grow in the YPD medium containing 47.34, 63.12, and 78.9 g/L ethanol. A slight decrease in growth was observed when it was grown in 94.68 g/L ethanol, whereas it was completely inhibited at 110.46 g/L ethanol. Consistent with our findings, Chamnipa et al. [13] stated that the strain *P. kudriavzevii* RZ8–1 could withstand an ethanol concentration of up to 94.68 g/L on Yeast Malt agar at 35 °C. Likewise, Tikka et al. [42] reported that *S. cerevisiae* yeast grew in the presence of 94.68 g/L ethanol. However, Pongcharoen et al. [43] revealed that three strains of *P. kudriavzevii* NUNS-4, NUNS-5, and NUNS-60 were able to tolerate 102.57 g/L ethanol, which is higher than the results obtained in this study.

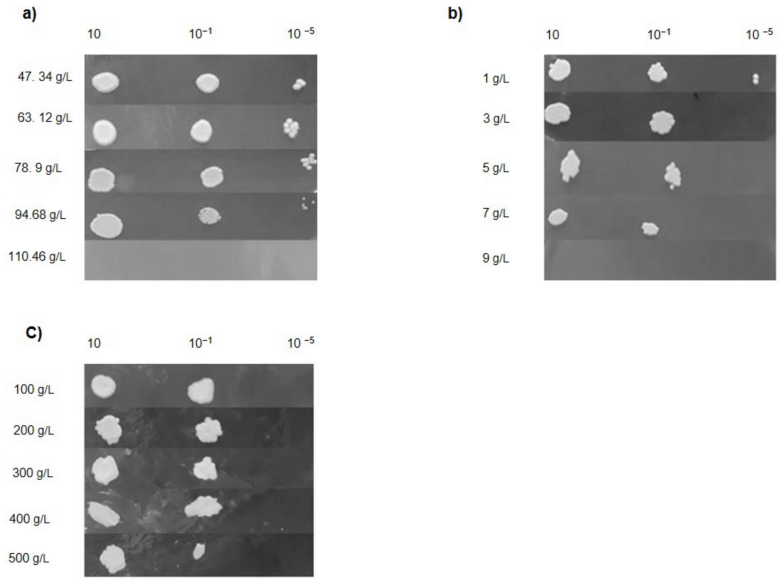

Figure 2. Effect of (**a**) ethanol, (**b**) acetic acid, and (**c**) glucose concentrations on cell growth of *P. kudriavzevii* YSR7.

Acetic acid is one of the main microbial-inhibiting substance found in lignocellulosic biomass hydrolysates [44]. Numerous studies indicated that important titers of acetic acid strongly affect ethanol fermentation. It reduces the cytoplasmic pH of cells and blocks the activity of enzymes, particularly that of aldolase, endolase, phosphoglyceromutase, and triosephosphate isomerase [45–47]. As presented in Figure 2b, the strain grew well in YPD containing concentrations of 1, 3, 5, and 7 g/L of acetic acid. Instead, its growth was completely inhibited at the concentration of 9 g/L. These observations are in agreement with those made by Chamnipa et al. [13] and Favaro et al. [48], who reported that the growth of *P. kudriavzevii* RZ8–1 and some strains of *S. cerevisiae*, respectively, was barely visualized in a medium containing 7.5 g/L acetic acid.

Osmotic pressure exerted by high concentrations of sugars during ethanol fermentation generates a loss in the osmotic gradient across the plasma membrane of yeasts and disrupts the function of genes involved in the stress response, resulting in decreased cell viability [49,50]. As shown in Figure 2c, the yeast *P. kudriavzevii* YSR7 was able to tolerate high osmotic pressure of up to 500 g/L of glucose. Similarly, Rodrussamee [51] stated that three yeasts named *P. manshurica* MY2/P1, *P. kudriavzevii* S/PA1 and *Starmerella bacillaris* MY1/P3 could grow at a glucose concentration of up to 450 g/L.

4. Conclusions

In this study, efficient thermotolerant ethanologenic yeasts were successfully isolated from natural fermented products (Sourdoughs, Raib, Leben, and Smen). Among these isolates, a yeast identified as *P. kudriavzevii* YSR7 exhibited promising potential for ethanol fermentation at high temperatures. This strain assimilated and fermented glucose and xylose at elevated temperatures of up to 45 °C. In addition, ethanol concentrations of 22.36 g/L from BS, 18.2 g/L from CS, and 6.34 g/Lfrom OTP were produced, respectively, through a simultaneous saccharification and fermentation process at 42 °C. It also tolerated high concentrations of ethanol, acetic acid, and glucose. Our study provided important data about the use of *P. kudriavzevii* YSR7 for ethanol production from lignocellulosic biomass.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en15144954/s1, Table S1: Fermentation capacity test of thermotolerant yeasts isolates on YPD broth containing Durham tubes and 31.56 g/L ethanol.

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